Insights into the mechanism of action of cidofovir and other acyclic nucleoside phosphonates against polyoma- and papillomaviruses and non-viral induced neoplasias

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Abstract

Acyclic nucleoside phosphonates (ANPs) are well-known for their antiviral properties, three of them being approved for the treatment of HIV (tenofovir), chronic hepatitis B infections (tenofovir and adefovir) or human cytomegalovirus retinitis (cidofovir). In addition, cidofovir is mostly used off-label for the treatment of viral infections caused by several DNA viruses other than cytomegalovirus, including papilloma- and polyomaviruses, which do not encode for their own DNA polymerases. There is considerable interest in understanding why cidofovir is effective against these small DNA tumor viruses. Considering that papilloma- and polyomaviruses cause diseases associated either with productive infection (characterized by high production of infectious virus) or transformation (where only a limited number of viral proteins are expressed without synthesis of viral particles), it can be envisaged that cidofovir may act as antiviral and/or antiproliferative agent.

The aim of this review is to discuss the advances performed during the last years in understanding the mode of action of ANPs as antiproliferative agents giving the fact that current data suggest that their use can be extended for the treatment of non-viral related malignancies.

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1. Introduction

The acyclic nucleotide analogue cidofovir {(CDV), 1-[(*S*)-3-hydroxy-2-(phosphonylmethoxy)-propyl]cytosine, HPMPC}, displays potent activity against a broad spectrum of DNA viruses. The intravenous formulation of CDV has been formerly licensed for the treatment of human cytomegalovirus (HCMV) retinitis in AIDS patients in 1996. However, this compound is mostly used off-label for the treatment of severe infections caused by various DNA viruses other than HCMV (De Clercq, 2007,De Clercq, 2011). Different formulations of CDV have been employed for the management of acyclovir resistant and/or foscavir resistant herpes simplex virus infections, poxvirus-associated diseases including molluscum contagiosum virus and orf virus, life-threatening adenovirus and human polyomavirus (PyV) infections as well as human papillomavirus (HPV)-associated hyperproliferative diseases. A summary of the applications of CDV as an antiviral and antiproliferative agent in the treatment of human diseases is presented in **Table 1**.

CDV belongs to the class of acyclic nucleoside phosphonates (ANPs), which are well-known for their antiviral properties. In addition to CDV, two other ANPs got approval for the treatment of viral infections (De Clercq and Holy, 2005,De Clercq, 2007,De Clercq, 2006). Tenofovir {PMPA, (*R*)-9-[2-(phosphonylmethoxy)propyl]adenine} and adefovir {PMEA, 9-[(2-phosphonylmethoxy)ethyl]adenine} are active against retro- and hepadnaviruses, their oral prodrugs forms being licensed for the therapy of human immune deficiency virus (HIV) (tenofovir) and of chronic hepatitis B virus (HBV) infections (tenofovir and adefovir).

ANPs have been shown to enter the cell by an endocytosis-like process and they are converted intracellularly to their diphosphate metabolites by cellular enzymes (De Clercq and Holy, 2005). The diphosphate forms of the ANPs (i.e. CDVpp, PMEApp and PMPApp) interact as competitive inhibitors/alternative substrates with respect to the normal substrates (i.e. dCTP and dATP). Incorporation of one molecule of PMEApp or PMPApp into the growing DNA strand results inevitably in DNA chain termination whereas CDVpp requires two consecutive incorporations to efficiently terminate DNA synthesis, as has been shown for HCMV (Xiong et al., 1996,Xiong et al., 1997). The selective antiviral activity of ANPs results from the higher affinity of the ANPpp for viral DNA polymerases [that is herpesvirus and poxvirus DNA polymerases and HIV or HBV reverse transcriptases] than for cellular DNA polymerases α, δ, and ε. **Figure 1** illustrates the intracellular activation of CDV and its mode of action against viruses encoding for their own DNA polymerases. The mechanism of action of ANPs as antiviral agents has been extensively summarized in various reviews (De Clercq, 2003,Andrei and Snoeck, 2010,De Clercq, 2007,De Clercq, 2011,De Clercq and Holy, 2005) and will not be further discussed here.

Besides their well-recognized antiviral characteristics, CDV as well as some PME derivatives, such as PMEA, PMEDAP {9-[(2-phosphonylmethoxy)ethyl]-2,6-diaminopurine} and PMEG {9-[(2-phosphonylmethoxy)ethyl]guanine} **(Figure 2)**, possess antiproliferative properties, although their mechanisms of antitumor efficacy appear to be dissimilar considering that CDV is not an obligate chain terminator, in

contrast to the PME derivatives, and that the effects of CDVpp on cellular DNA polymerization are weaker compared to the diphosphate forms of the PME derivatives (Wolfgang et al., 2009).

In this review, we focus on the antiproliferative activities of ANPs and we debate on their mode of action against viruses, such as polyomaviruses (PyVs) and papillomaviruses (PVs) that do not encode for their own DNA polymerases. Also, the potential use of ANPs for the treatment of non-viral induced tumors will be discussed.

2. Similarities and differences between polyomaviruses (PyVs) and papillomaviruses (PVs)

Until 2000, PVs and PyVs were grouped together in the family Papovaviridae ("pa-po-va" stands for papilloma- polyoma – vacuolizing agent SV40). Since then, the family Papovaviridae is obsolete and the Papillomaviridae and Polyomaviridae families were recognized by the International Committee on Taxonomy of Viruses (ICTV) (Johne et al., 2011,de Villiers et al., 2004)

Table 2 summarizes the main similarities and differences between PyVs and PVs. These two viral families have a non-enveloped icosahedral capsid (composed of 72 capsomers) surrounding a double-stranded circular DNA genome of ~5kbp in PyVs and of ~8kbp in PVs. Both viruses use overlapping genes and differential splicing to pack the maximum amount of genetic material in the minimum space. All open reading frames (ORFs) are located on only one (PVs) or both (PyVs) strands of DNA, as depicted in Box 1 and Box 2, respectively. Two classes of genes, the early (E) genes (which are required for viral DNA replication) and late (L) genes (coding for the structural proteins) exist in both PyVs and PVs.

The HPV genome contains a coding region that encompasses an E region that includes up to seven ORFs encoding non-structural proteins and the late region comprises the L1 and L2 ORFs. In HPV, a ~1kbp non-coding region [also known as the long control region (LCR) or the upstream regulatory region] separates the early and late regions. The LCR harbours the origin of replication, the transcription start sites and promoter/enhancer elements that regulate viral gene expression. In PyV, both strands of DNA code for the viral proteins. One strand of DNA encodes an overlapping set of multifunctional early regulatory proteins and the other strand encode for the capsid proteins expressed late in permissive cells. Some PyVs also encode for an agno protein that facilitates virion assembly. The control region between the early and the late transcription units contains a bidirectional enhancer, early and late promoters, the viral origin of replication, the viral packaging signal and binding sites for host transcription factors.

Papillomavirus particles are ~55 nm diameter, compared to ~45 nm diameter in PyVs. Papillomaviruses encode two structural proteins: the major capsid protein, L1 (~510 amino acids and ~58 kDa), and the minor protein L2 (~470 amino acids and ~51 kDa). In contrast, PyVs encode for three structural proteins: the major capsid protein, VP1 (~370 amino acids and ~41 kDa) and two minor proteins VP2 (~350 amino acids and ~38 kDa) and VP3 (~230 amino acids and ~26 kDa). Despite significant differences in amino acid sequences of the major capsid proteins, both PV and PyV capsids exhibit conserved features, as the 72 capsomers are pentamers

of the major capsid protein and are arranged on a T=7 icosahedral lattice. Papillomaviridae and Polyomaviridae differ in capsomer morphology and size. Papillomavirus capsomers are star-shaped, 11 to 12 nm in diameter, while polyomavirus are barrel-shaped, 8 nm in diameter. Intercapsomer interactions are also slightly different between these viral families (Belnap et al., 1996). The carboxyl terminus of VP1 or L1 mediates contacts between the pentamers in the capsid. While disulfite bonds stabilize the interpentamer contacts for L1, both disulfite bonds and calcium bridges stabilize these contacts for VP1 (Sapp and Day, 2009). Also, differences in receptor binding and internalization pathway also exist between PVs and PyVs, reviewed in (Sapp and Day, 2009).

Polyomaviruses generally have a narrow host range and limited cell type tropism (Gjoerup and Chang, 2010). In their natural host, they are able to infect cells giving rise to a productive life cycle causing cell lysis. In addition, PyVs establish a latent/persistent infection that is asymptomatic and is rarely associated with disease unless when the immune system is impaired. The full, infectious viral life cycle of human PyVs has only been studied for JCPyV and BKPyV because no infectious system exists up to now for the other human PyVs **(Box 3)**. As PyVs are non-enveloped viruses, the viral capsid proteins interact directly with the receptor molecules in order to gain entry into the cells, being this interaction a major determinant of host and tissue tropism. Entry of PyVs into the cells includes receptor binding, internalization and intracellular trafficking, virus uncoating and nuclear entry. Once the uncoated viral genome is inside the cells, the regulatory early proteins [Large tumor antigen (LT-ag) and small T antigen (sT-ag) are produced in all PyVs. Besides LT-ag and sT-ag, other virus-specific T-antigen isoforms [such as middle T antigen (mT-ag) in rodent PyVs, the 17kT antigen in SV40 and the 57kT antigen in Merkel cell polyomavirus (MCPyV)] are derived from alternative splicing of the LT-ag transcript (Cheng et al., 2009,An et al., 2012,Topalis et al., 2013). Some PyVs can cause tumors and products from the early region, especially SV40 LT-ag and murine PyV mT-ag, are required for cellular transformation.

In benign lesions induced by PyVs, viral genomes are typically maintained extra-chromosomally. Malignant progression, as in the case of Merkel cell carcinoma (MCC), is associated with viral integration into host cell chromatin **(Box 1)**. Although MCPyV is very common, MCC is very infrequent, most probably because integration is not part of the MCPyV life cycle and is a rare event. This integration event is involved in the initiation of the tumor, since MCPyV was found to be clonally integrated into a single site of the host genome, indicating that viral integration preceded tumor expansion (Feng et al., 2008,DeCaprio and Garcea, 2013). Recently, an overprinting gene, expressed from an Alternate Frame of the Large T Open reading frame (ALTO) was identified in MCPyV (Carter et al., 2013). Although ALTO is expressed during replication of MCPyV genome it is not required for replication. Despite no sequence similarities with the rodent mT-Ag, ALTO was found to be evolutionary related to mT-ag.

Both PyV and PVs multiply in the nucleus of the infected cell and their circular genome associates with host encoded histones in the virions. These small DNA tumor viruses widely rely on the host cell DNA replication

machinery to replicate their genomes. The LT-ag in PyVs is a multifunctional initiator protein that can successively recognize the viral origin of replication, assemble into a double hexamer melting and unwinding the DNA ahead of the replication fork, and interact with the host DNA replication factors (such as polymerase α-primase, replication protein A (RPA) and topoisomerase I (**Box 4**). The LT-ag also contains several intrinsic biochemical activities and binds to several cellular proteins, directing the cellular machinery to support viral replication. LT-ag interacts with heat shock protein 70 (Hsc70) through its DnaJ domain and with members of the retinoblastoma (Rb) family of pocket proteins (i.e. pRB, p107, and p130) through the LXCXE motif in its N-terminal region. Binding of LT-ag to the Rb family of proteins impairs their role as repressor of E2F transcription factors promoting transition into S-phase of the cell cycle **(Box 5).** LT-ag also interacts with the tumor suppressor protein p53 and functionally inactivates its ability to induce cellular senescence or apoptosis in response to DNA damage (Cheng et al., 2009,Topalis et al., 2013,An et al., 2012). Thus, the LT-ag has pleiotropic functions, including initiation and maintenance of viral DNA replication, regulation of early and late genes transcription, virion assembly and manipulation of the host cell cycle through a number of protein-protein interactions. The LT-ag has also been shown to induce transformation and immortalization in different *in vitro* and *in vivo* models which can be attributed, in part, to the ability to inactivate the tumor suppressor proteins p53 and pRb.

The LT-ag is such a multifunctional protein that the immediate targets of interaction with host cell regulatory proteins are very difficult to unleash, even with experimental site-directed mutagenesis of this very large, multi-domain viral protein that forms 12 subunit homo-complexes as well as diverse hetero-complexes with various host proteins. Papillomaviruses carry out virtually the same interactions with the host cell as do PyVs, although PVs do so by using separate gene products. Therefore, the targets and functions of HPV early proteins (i.e. E6, E7, E1, and E2) are far more assignable than they are with large T-ag, which incorporates all these functions. Another source of misinformation when comparing PyVs with PVs is that almost all the biology of the PyVs has been studied using immortalized cell lines grown in monolayers, and many important interactions have been missed because the cells are constitutively activated for pathways normally targeted for activation (or suppression) by the viruses in living host organisms.

LT-ag is indispensable for PyV DNA replication which begins when two hexamers of the LT-ag are formed in a head-to-head orientation at the origin of replication. Most organisms have a replicative DNA helicase that unwinds DNA as a single hexamer that encircles and translocates along one strand of the duplex DNA and excludes the complementary strand (known as steric exclusion). It has been a matter of debate whether a single or a double hexamer of LT-ag encircles and acts on single-stranded DNA or double-stranded DNA during unwinding. A recent study has clearly shown that a double hexamer of LT-ag assembles at replication origin, and then separates into two single hexamers and each hexamer unwinds dsDNA by encircling and translocating along each ssDNA in the 3'- to -5' direction **(Box 4)** (Yardimci et al., 2012). Furthermore, the authors described

the surprising ability of the LT-ag to overcome molecular barriers in its path by transiently opening its ring. This highlights the remarkable plasticity of hexameric structures.

Following recognition and binding to the origin of replication, melting of the DNA helix surrounding the origin, and oligomerization into two hexamers at the origin of replication, the LT-ag then recruits the cellular DNA replication factors: RPA, topoisomerase I and polymerase α primase. Type I topoisomerases are essential to relieve supercoiling stress as the strands unwind (Lin et al., 2002). Podophyllotoxin (Condylox) is a topoisomerase I inhibitor in clinical use against HPV lesions to block viral DNA replication (Stern et al., 2012). As podophyllotoxin is also active against host chromosomal replication, it is cytotoxic. Following the initiation events, the clamp loader, replication factor C (RFC), and the polymerase processivity factor, PCNA (proliferating cell nuclear antigen), are recruited and loaded leading to the binding and activity of DNA polymerase δ, which extends both lagging and leading strands. After PyV DNA replication and the expression of late structural proteins, new progeny virions are assembled and are released from the infected cell.

Papillomaviruses are highly diverse and have been discovered in a wide array of vertebrates and their host range include all amniotes (Rector and Van Ranst, 2013). Papillomaviruses are highly host-restricted, and cause abortive infections in non-host species. The HPV life cycle is closely linked to the differentiation state of the epithelial cells and the initial step involves the infection of keratinocytes in the basal layer of squamous epithelia **(Box 6)** (Stanley, 2012,Chow et al., 2010,Duensing and Munger, 2004,zur Hausen H., 2002). Similarly to PyVs, HPVs do not encode for their own DNA polymerases but they encode for viral proteins (i.e. E1 and E2) that are required for viral genome replication during the HPV productive cycle **(Box 7).** (D'Abramo and Archambault, 2011,McBride, 2013,Bergvall et al., 2013). E1 is the most highly conserved HPV protein and the only one with enzymatic activity. E1 is the replicative helicase of HPV and is essential for viral replication and pathogenesis. Both LT-ag and the E1 protein are structurally related members of the helicase superfamily III (SF3). E1 binds to the origin of replication together with E2 protein. In fact, the E2 protein assists and directs faithful viral origin recognition of E1 while E1 is the replicative DNA helicase, melting the DNA around the origin of replication and establishing itself as a double hexameric helicase. The formation of the E1-E2-origin of replication complex involves not only the binding of E1 and E2 to specific viral DNA elements in the origin of replication but also a protein-protein interaction between the N-terminal transactivation domain of E2 and the helicase/ATPase domain of E1 **(Box 7)**. Similar to LT-ag, E1 also acts to recruit the cellular DNA replication proteins to the PV DNA replication fork.

HPV E6 and E7 genes encode low molecular weight proteins of about, respectively, 150 and 100 amino acids (**Box 8**). It has been shown that expression of E6 and E7 from high-risk HPV types is necessary and sufficient to immortalize primary keratinocytes, abrogates DNA damage responses, causes genomic instability, and induces epithelial cell hyperplasia (Ghittoni et al., 2010,Hellner and Munger, 2011,Moody and Laimins, 2010). HPV E6 and E7 proteins do not have intrinsic enzymatic activity but function by associating with several cellular proteins

resulting in the alteration of various host cellular pathways. Specific interactions of E6 and E7 with key cell cycle regulatory proteins [namely E6 with the tumor suppressor protein p53 and E7 with the Rb family of pocket proteins] are responsible for the potential oncogenicity of the high-risk HPV types **(Box 9)**. An important function of p53 is to induce the expression of genes that alter cell cycle progression in G1/S phase in response to DNA damage. Crucial host cell targets of the high-risk E6 protein include many PDZ domain-containing proteins involved in cell-cell contact, communication and polarity (Howie et al., 2009). The Rb family of proteins control the transition at the G1/S phase of the cell cycle by binding and regulating the activity of the E2F family of transcription factors. As a consequence of these interactions, E7 stimulates quiescent cells to re-enter S-phase while E6 prevents cellular growth arrest or DNA-damage induced apoptosis **(Box 9).**

In contrast to PyV LT-ag that inactivates Rb/E2F complexes by stoichiometric association with Rb, high-risk HPV E6 and E7 proteins target, respectively, p53 and Rb for ubiquitin-mediated proteosomal degradation (Pim and Banks, 2010,McLaughlin-Drubin and Munger, 2009,Yugawa and Kiyono, 2009,Moody and Laimins, 2010,Miller et al., 2012). E6 associates with the cellular E3 ubiquitin ligase E6-associated protein (E6AP) and the E6/E6AP complex binds p53 and induces its specific ubiquitinylation and subsequent degradation by the proteasome (**Box 9**). High-risk HPV E7 mediates degradation of Rb by a mechanism involving association with and reprogramming of the cullin 2 (CUL2) ubiquitin ligase complex, resulting in the release of active E2F transcription factor which in turn activates the transcription of genes encoding proteins (such as cyclin E and cyclin A) necessary for cell cycle progression (**Box 9**).

One member of the RB family, p130, appears to be an important target for E7 in promoting its proteosome-mediated destruction and S-phase entry. Recent evidence indicates that p130 regulates cell-cycle progression as part of a large complex named DREAM (DP, Rb-like, E2F and MuvB). In addition, it was demonstrated that high-risk HPVs can bind to MuvB core complex and activate gene expression during the G2 and M-phase of the cell cycle. Thus, high-risk HPV E7 perturbs the DREAM complex to prevent exit from the cell cycle entry and also to promote cellular proliferation and mitotic gene expression (DeCaprio, 2014,Banerjee et al., 2011,Nor et al., 2013,Nor et al., 2011).

E4 and E5 proteins contribute indirectly to genome amplification success because they modify the cellular environment. E5 is a small transmembrane protein with a cytoplasmatic C-terminus (**Box 8**). It is thought to function by inducing ligand-independent dimerization and activation of receptor protein tyrosine kinases, including the epidermal growth factor receptor (EGFR) (DiMaio and Petti, 2013). Hence, E5 contributes to genome amplification success through its ability to stabilize EGFR and its role in up-regulation of mitogenic signal transduction. Many but not all HPVs encode for E5, and this viral oncoprotein contributes to some early steps of viral transformation but it is not necessary for malignant progression and/or maintenance of the transformed phenotype since E5 is not generally expressed in cervical carcinomas. While bovine papillomavirus (BPV)-1 E5 protein interacts with PDGF (platelet derived growth factor), this is not an activity of the HPV E5 protein. BPV-1

E5 protein (which functions as a disulfide cross-linked dimer) is phylogenetically unrelated to the E5 proteins of alpha group HPV types (which form hexameric transmembrane pores, placing it within the virus-encoded "viroporin" family). It was found that high-risk human papillomavirus E5 oncoprotein displays channel-forming activity sensitive to small-molecule inhibitors (Wetherill et al., 2012).

The productive phase of the HPV life cycle occurs in the terminally differentiated layers of the stratified epithelium, where viral particles are assembled and shed. Differentiation of infected cells induces genome amplification and a remarkable increase in late gene expression resulting in packaging of the viral genome and virus release (Doorbar et al., 2012). The E4 protein is abundantly expressed in the upper epithelial layers in cells that support viral genome amplification. E4 is primarily involved in some aspect of virus release or transmission, as it was shown to induce the disruption of keratin structure, and in promoting proper viral assembly (Doorbar et al., 1991,Wang et al., 2004).

During the productive HPV life cycle, the genome is maintained as an episome but in almost all high-grade lesions and tumors, the viral genome is integrated into the host genome. The viral oncoproteins E6 and E7 are expressed in high-grade intraepithelial neoplasias associated with HPV infection (Bodily and Laimins, 2011,Doorbar et al., 2012). Expression of E6 and E7 is transcriptionally regulated by E2 during the productive HPV life cycle. In cancer progression, the integration of the viral genome occurs in such a way that disruption of the E2 open reading frame occurs eliminating the E2-mediated transcriptional control of the early viral region **(Box 2),** leading to constitutive expression of E6 and E7 proteins in HPV-associated cancers. The continuous expression of these two viral oncoproteins contributes to the maintenance of proliferation and malignant phenotypes of the cancer cells due to their disruptive action on cell cycle checkpoint. Therefore, E6 and E7 are considered to be potential therapeutic targets for blocking the development of HPV-related cancer. Ideally, small molecules that target and prevent the interaction of E6 and E7 with cellular proteins may have interesting antiproliferative potential (Manzo-Merino et al., 2013). Besides E6 and E7, part or all of E1 is transcribed and translated in neoplasias. The amino-terminal portion of E1 protein or a truncated peptide is essential to bind to and neutralize over-abundant cyclins that are transcriptionally up-regulated by E7 (Stoler et al., 1992,Lin et al., 2000,Coupe et al., 2012).

3. Clinical impact of human polyomaviruses

The name polyomavirus is derived from the ability of the first PyV discovered more than 50 years ago to induce multiple (poly) tumors (oma) in mice. However, most PyVs do not cause tumors in their natural host. Mouse polyomavirus (MPyVs) and the simian vacuolating agent 40 (SV40) were the first PyVs identified (Atkin et al., 2009). Two human PyVs were identified in 1971 and were named following the patients' initials from whom they were isolated [JC polyomaviruses (JCPyV) was identified in a brain tissue extract from a patient (John Cunningham) with progressive multifocal leukoencephalopathy (PML) and BK polyomavirus (BKPyV) was isolated

from the urine of a nephropathic kidney transplant patient of unknown name] (Dalianis and Hirsch, 2013,Hirsch et al., 2013,Gjoerup and Chang, 2010). Subsequently, more PyVs were identified in mammals and birds. From 2007 on, several new human PyVs have been discovered, including KI (Karolinska Institutet) virus (KIPyV), WU (Washington University) virus (WUPyV), Merkel cell polyomavirus (MCPyV), HPyV6, HPyV7, HPyV9, *Trichodysplasia spinulosa* virus (TSPyV), HPyV10 [Malawi virus (MWPyV and MX polyomavirus (MXPyV) variants], HPyV12 and Saint Louis Polyomavirus (STLpYV) (Van Ghelue et al., 2012,Pastrana et al., 2013,Ehlers and Wieland, 2013,Yu et al., 2012,Feltkamp et al., 2013,White et al., 2013).

Serological studies indicate that human PyVs sub-clinically infect the general population with rates ranging from 35% to 90%, and significant disease is only observed in patients with impaired immune functions (Dalianis and Hirsch, 2013,Chang and Moore, 2012). Thus, BKPyV has been linked to hemorrhagic cystitis (HC) after allogenic hematopoietic stem cell transplantation and PyV-associated nephropathy (PyVAN) after kidney transplantation, while JCPyV is associated with PML in HIV-AIDS, haematological diseases and in autoimmune diseases treated with certain lymphocyte-specific antibodies (Dalianis and Hirsch, 2013,Bennett et al., 2012,Jiang et al., 2009).

TSPyV was identified in *Trichodysplasia spinulosa*, a rare skin disease characterized by virus-induced lytic as well as proliferative tumor-like features observed in immunosuppressed transplant recipients (Kazem et al., 2012,Wanat et al., 2012,van der Meijden E. et al., 2010,Kazem et al., 2013). MCPyV is associated with a rare skin cancer, Merkel cell carcinoma (MCC), seen in the elderly and in chronically immunosuppressed individuals (Spurgeon and Lambert, 2013,Arora et al., 2012). MCPyV is found in at least 80% of MCC and clonal viral integration and truncating mutations of the Large T antigen (LT-ag) support an etiopathogenic role of MCPyV in MCC (Feng et al., 2008,Rodig et al., 2012,Shuda et al., 2008). MCPyV might not be exclusively linked to the development of MCC. The presence of MCPyV DNA has been evaluated in a variety of other cancers since this virus was linked to MCC (Spurgeon and Lambert, 2013). A potential role of MCPyV in a significant subset of chronic lymphocytic leukemia (CLL) is claimed based on a study performed on 70 patients (Pantulu et al., 2010). The authors demonstrated a relative high incidence of MCPyV in highly purified CLL cells in 27.1 % of patients and the presence of a truncating LT-ag deletion in 8.6% of CLL cases. Thus, MCPyV may represent the molecular correlate of the long term recognized epidemiologic association of CLL and MCC and vice versa. Additionally, contradictory reports have been published on the relationship between squamous cell carcinoma (SCC) and MCPyV. Some groups have found no significant association (Andres et al., 2010,Reisinger et al., 2010) whereas others found virus DNA in 40% of cutaneous SCC (Kaibuchi-Noda et al., 2011,Rollison et al., 2012).

In contrast, KIPyV and WUPyV (found in the respiratory tract), HPyV6 and 7 (present in the skin), and HPyV9 (isolated from serum and skin), MWPyV, STLPyV and HPyV12 (found in stool samples) have so far not been linked to any disease (Ehlers and Wieland, 2013).

Infections with human PyVs occur early in life leading to a primary viremia followed by a state of latency/persistence and escape from the immune system. The site and the molecular nature of viral latency/persistence are not fully understood and differs among human PyVs (White et al., 2013). They can persist in the host cells in the absence of viral replication, i.e. a state of viral latency, for example JCPyV in the brain. Alternatively, human PyVs may persist in a state of active but asymptomatic viral replication, as it is the case for JCPyV and BKPyV in the kidney.

4. Clinical importance of HPVs

Papillomaviruses have a tropism for squamous epithelia and today, 165 HPV types have been described (Burk et al., 2013,Bernard et al., 2010), the number is growing as more types are officially classified. Although various HPV types have a comparable genomic organization, different HPVs infect mucosal or cutaneous epithelia at distinct body locations. About 48 of the 165 different HPV types are able to infect the anogenital and oral mucosa, and they can be further classified into low-risk and high-risk types based on their potential to induce cellular transformation. Low-risk types cause benign epithelial proliferation (warts), while infection with high-risk types may lead to cancer progression. HPV6 and 11 are the most abundant low-risk types, causing more than 90% of condylomata acuminata (genital warts) (Doorbar et al., 2012). Recurrent respiratory papillomatosis (RRP) is also caused by low-risk HPV types (mostly HPV6 and 11). HPV infection leading to RRP occurs mostly during vaginal delivery but HPV DNA detection in amniotic fluid, foetal membranes, cord blood and placental trophoblastic cells suggest that HPV infection can also take place *in utero*, i.e. prenatal transmission (Syrjanen, 2010). Recurrent respiratory papillomatosis can also arise later in life and, indeed, about half of all RRP cases first show up in adults. (Derkay and Wiatrak, 2008).

In 2008, H. zur Hausen was awarded the Nobel Prize of Physiology or Medicine because of his research on the association between high-risk HPV types with premalignant cervical lesions and cancer (zur Hausen H., 2002). Virtually 100% of cervical cancers contain HPV DNA sequences from a high-risk oncogenic HPV type, HPV16 and 18 being found in about 70% of cases. Besides cervical cancer, HPVs are associated with a number of other anogenital cancers, including vulvar, vaginal, penile and anal cancers. HPV-associated anogenital cancers are preceded by a spectrum of intraepithelial abnormalities, ranging in the case of the cervix from low-grade CIN (cervical intraepithelial neoplasia) 1, moderate CIN2 and high-grade CIN3 (Hellner and Munger, 2011,Cubie, 2013). Genital infections with high-risk HPV types are very common among sexually active individuals and although the majority of them clear the infection with time, a proportion of women (approximately 15%) cannot eliminate the virus and persistence with a high-risk HPV type is considered the major risk factor for the development of malignancies.

High-risk HPVs are also found in a proportion of head and neck squamous cell carcinomas (HNSCC) and it is recognized that HPV-positive HNSCC present a different biology than that of HPV-negative HNSCC (Miller et al.,

2012,Leemans et al., 2011). Recent studies have shown that the incidence of HPV-negative HNSCC has decreased as a consequence of public efforts encouraging smoking cessation and reduced consumption of alcohol, in contrast to HPV-positive HNSCC whose incidence is increasing (most likely due to changes in sexual behaviour) (Olthof et al., 2012,Rietbergen et al., 2013).

5. PME derivatives

5.1 *In vitro* and *in vivo* antiproliferative activities

PMEG was studied for effectiveness against cotton tail rabbit papillomavirus (CRPV) infection of rabbits and HPV11 infection of human foreskin xenografts in athymic mice (Kreider et al., 1990). PMEG strongly suppressed the growth rates of Shope papillomas and inhibited HPV11 infections of human skin. Although drug toxicity paralleled the therapeutic effects in rabbits, there was much less toxicity in athymic mice.

Three phosphonomethoxyalkyl purine analogues, i.e. HPMPA {(S)-9-[(3-hydroxy-2-phosphonylmethoxy)propyl]adenine}, PMEA, and PMEG proved modestly active against intraperitoneal injected P388 murine leukemia cells in mice, PMEG being the most active and most potent of the three compounds (Rose et al., 1990). In this study, PMEG was also evaluated against subcutaneously implanted B16 melanoma in mice, affording increased life span and delay in primary tumor growth.

When the PME analogues PMEA, PMEDAP and PMEG were evaluated for their *in vitro* antitumor efficacy against human leukemia cells (Franek et al., 1999), they caused reversible slowdown of growth at low concentrations due to continuous repairing of damaged DNA, while high concentrations induced apoptosis and a reduction of the proportion of cells in the G1 phase of the cell cycle. The antitumor properties of these analogues increased in the order PMEA<PMEDAP<PMEG.

PMEG, PMEA, and PMEDAP were also investigated in a model of spontaneous T-cell lymphoma in inbred SD/cub rats (Otova et al., 1999). Treatment with 16 daily doses of PMEDAP at 5 mg/kg applied to the vicinity of the growing lymphoma resulted in significant therapeutic effects while daily PMEA or PMEG administration (although at lower doses than those of PMEDAP) did not affect survival of lymphoma-bearing mice. PMEDAP was shown to induce apoptosis in this *in vivo* model of hematological malignancies.

Because the utility of PMEG as an anticancer agent is limited by poor cellular permeability and toxicity (especially for the kidney and gastrointestinal tract), prodrugs such as N6-cyclopropyl-PMEDAP (cPr-PMEDAP), GS-9191 and GS-9219 **(Figure 2)** have been designed to increase permeability and accumulation of PMEGpp intracellularly (Kreider et al., 1990,Compton et al., 1999,Vail et al., 2009,Wolfgang et al., 2009). cPr-PMEDAP is converted to PMEG and can be considered as an intracellular prodrug of PMEG, limiting plasma exposure to the toxic agent PMEG. cPr-PMEDAP showed higher antitumor efficacy and selectivity in choriocarcinoma-bearing rats compared to PMEDAP or PMEG (Naesens et al., 1999) and was reported to have 8- to 20-fold more

pronounced cystostatic activity than PMEDAP and equivalent activity as PMEG against a variety of tumor cell lines (Hatse et al., 1999a).

GS-9191, a double prodrug of PMEG, was specifically designed as a topical agent to permeate the skin and to be metabolized to the active form in the epithelial layer. The conversion of GS-9191 to cPr-PMEDAP was shown to occur in lysosomes via carboxypeptidase cathepsin A-mediated ester cleavage, being cPr-PMEDAP subsequently translocated to the cytosol where it undergoes deamination and phosphorylation, yielding the active metabolite PMEGpp (Birkus et al., 2011). Recently, it was demonstrated that the adenosine deaminase-like (ADAL) protein plays a key role in the deamination of cPr-PMEDAP to produce PMEG, as mutations in this enzyme were shown to confer resistance to cPr-PMEDAP and its prodrugs but not to PMEG (Frey et al., 2013). On the other hand, resistance to both PMEG and cPr-PMEDAP was associated with a decreased capacity of the resistant cells to metabolically activate (phosphorylate) PMEG, resulting from amino acid substitutions in the guanylate kinase (involved in the conversion of PMEG to PMEGp) (Mertlikova-Kaiserova et al., 2011).

GS-9191 administered topically decreased the size of papillomas in a dose-dependent manner in an animal model of CRPV, affording the highest dose (0.1%) evident cures at the end of 5 weeks (Wolfgang et al., 2009). Based on these encouraging findings, topical GS-9191 was evaluated in a Phase II clinical trial (ClinicalTrials.gov Identifier: NCT00499967) for the treatment of genital warts in 2009 by Graceway Pharmaceuticals but the results of this trial have not been published (http://clinicaltrials.gov).

GS-9219, a phosphonoamidate prodrug of PMEG was designed as a cytotoxic agent that preferentially targets lymphoid cells *in vivo,* releasing PMEG in a two-steps process via enzymatic hydrolysis and deamination (Reiser et al., 2008). GS-9219 displayed considerable antiproliferative activity against activated lymphocytes and hematopoietic tumor cell lines while resting lymphocytes and solid tumor cell lines were less sensitive to the compound. GS-9219 showed substantial *in vivo* efficacy in five dogs with advanced-stage non-Hodgkin's lymphoma (NHL) after a single intravenous administration, with either no or low-grade adverse events (Reiser et al., 2008). In a Phase I/II trial conducted in pet dogs (n = 38) with naturally occurring NHL using different dose schedules of GS-9219, the compound was generally well tolerated and showed significant activity (Vail et al., 2009). Antitumor responses were observed in 79% of dogs and occurred in previously untreated dogs and dogs with chemotherapy-refractory NHL. Recently, GS-9219 (currently referred as VDC-1101) was evaluated against three human multiple myeloma (MM) cell lines, showing a dose-dependent antiproliferative activity (Thamm et al., 2014). In a Phase II clinical trial in dogs with spontaneous MM, major antitumor responses were observed in 9 of 11 evaluable dogs for a median of 172 days (Thamm et al., 2014).

Hostetler's group has synthesized alkoxyalkyl esters of PMEG and compared their antiproliferative activities with unmodified PMEG in primary human fibroblasts and CaSki, Me-180 and HeLa human cervical cancer cell lines *in vitro* (Valiaeva et al., 2010). Octadecyloxyethyl (ODE)-PMEG had excellent antiproliferative activity *in vitro* against the different human cervical carcinoma cell lines. In a Me-180 xenograft model in athymic nude

mice, intratumoral injection of 25 µg of ODE-PMEG or 100 µg of ODE-CDV daily for 21 days resulted in near-complete disappearance of measurable tumors, suggesting that ODE-PMEG may be suitable for local or topical treatment of cervical dysplasia.

5.2 Mechanism of antiproliferative effects of PME derivatives

As mentioned above, to exert their antiviral or antiproliferative activity, the PME derivatives need to be converted by cellular enzymes to their diphosphate forms. Studies performed with purified viral and cellular enzymes showed that the diphosphate metabolites effectively compete with the corresponding deoxynucleoside triphosphate (dGTP or dATP) for incorporation into DNA. As the diphosphate forms of PME derivatives are recognized as substrates by cellular DNA polymerases, they are able to inhibit cellular DNA synthesis by a direct inhibition of replicative cellular DNA polymerases. Indeed, a close correlation between cytostatic activities of PME derivatives and the inhibitory effects of their active metabolites on cellular DNA polymerases α, δ, and ε was established, emerging PMEG as the most potent chain terminating inhibitor of cellular DNA polymerases (Kramata et al., 1996,Kramata et al., 1998). Thus, the primary mechanism of action of PMEG in replicating cells is incorporation of its active metabolite PMEGpp into DNA and subsequent chain termination due to the lack of a 3'-hydroxy moiety.

Of note, PMEGpp was found to be more efficiently incorporated into DNA by DNA polymerases α and δ than by DNA polymerases β, γ, and ε (Kramata et al., 1996,Kramata et al., 1998). The interaction of PMEGpp with purified rat pol α, β, and δ, bovine pol δ and human pol ε were investigated by using oligonucleotide template-primers and by examining the inhibitory effects of PMEGpp and the ability of these enzymes to incorporate the analogue into DNA as well as to excise it from 3'-ends. DNA polymerases α (associated with primase activity) and δ are required for DNA synthesis of, respectively, the lagging strand and the leading strand of chromosomal DNA while DNA polymerase ε is required as a second DNA polymerase on the lagging DNA strand. In contrast to DNA polymerase α, both DNA polymerases δ and ε have intrinsic 3'-5'-exonuclease activity associated with a proofreading function and are necessary for the repair of DNA damage. While both enzymes can recognize PMEGpp as a substrate and can incorporate PMEG into DNA, DNA polymerase ε but not δ was shown to be able to repair the incorporated analogue (Kramata et al., 1998).

Wolfgang and collaborators investigated the mechanism of inhibition of PMEG and its prodrug GS-9191 against HPV (Wolfgang et al., 2009). Inhibition of DNA polymerases by PMEGpp was proposed as the prevailing mechanism of action, and this activity alone may explain their antiproliferative activity against cervical carcinoma HPV positive cells. Treatment of cells with these drugs resulted in inhibition of DNA synthesis and S-phase arrest leading to apoptosis induction. Thus, PMEG and GS-9191 preferentially affect rapidly dividing HPV-transformed cells (compared to normal keratinocytes, the majority of which are quiescent) because the inhibition of chromosomal DNA replication affects only cells in the S-phase of the cell cycle. In conclusion, due to the lack of an HPV viral polymerase, the active metabolite PMEGpp exerts its effect by inhibiting host cell

polymerases, and *in vivo*, partial selectivity for virally infected cells is likely derived based on the increased proliferation of infected cells and due to the fact that the compounds are locally administered.

Kramata and collaborators demonstrated that differences in inhibition of cellular DNA synthesis by PMEG, PMEDAP, and PMEA may be explained not only by different affinities of DNA polymerases (primarily DNA polymerase δ) for the nucleotide analogues but also by different intracellular ratios of the diphosphate analogues to their corresponding deoxynucleoside triphosphates (Kramata et al., 1996). Treatment of the human T lymphoblast cell line CEM with PMEG, PMEDAP or PMEA resulted in increased deoxynucleotide triphosphate (dNTP) pools, with PMEG producing the greatest increase. Although no significant differences in cellular uptake were found for these ANPs, CEM cells were found to accumulate higher levels of PMEGpp than PMEDAPpp or PMEApp, pointing also to differences in the efficiency of phosphorylation among these nucleotide analogues (Pisarev et al., 1997). It is interesting to note that more PMEGpp than PMEApp are produced considering that there is much more adenylate kinase than guanylate kinase in the cells resulting in more ADP/ATP than GDP/GTP. The investigations carried out by Pisarev and colleagues also highlighted that the factors contributing to the enhanced antileukemic activity of PMEG derives both from its increased anabolic phosphorylation and the increased potency of PMEGpp to target the cellular DNA polymerases compared to other PME analogues.

PMEA proved to be a strong inducer of differentiation of the erythroleukemia K562 cell line, as evidenced by hemoglobin production, increased expression of glycophorin A on the cell membrane, and induction of acetylcholinesterase activity (Hatse et al., 1999b). After exposure to PMEA, K562 cell cultures displayed a marked retardation of S-phase progression, leading to a severe perturbation of the normal cell cycle distribution pattern with marked accumulation of cyclin A and, most strikingly, cyclins E and B1. A similar effect on cell cycle deregulation was also observed in PMEA-exposed human myeloid THP-1 cells but, in contrast to the strong differentiation-inducing activity of PMEA in K562 cells, the drug completely failed to induce monocytic maturation of THP-1 cells. On the contrary, THP-1 cells underwent apoptotic cell death in the presence of PMEA. These data suggested that, depending on the nature of the tumor cell line, PMEA can trigger a process of either differentiation or apoptosis by affecting cell cycle processes through inhibition of DNA replication during the S phase.

Among several diphosphates of ANPs tested in a telomeric repeat amplification protocol (TRAP) for their ability to inhibit the extension of telomeres by human telomerase, the diphosphates of PMEG and PMEDAP emerged as the most potent drugs (Hajek et al., 2005). In this study, the ability of well-known inhibitors of the HIV reverse transcriptase to interfere with telomerase activity was investigated as the human telomerase active site (i.e. hTERT) was shown to function as a reverse transcriptase. However, the most potent chain-terminating inhibitors of retroviral reverse transcriptase (such as PMPApp and PMPDAPpp) did not inhibit human telomerase activity. In fact, PMEGpp (IC_{50} 12.7 ± 0.5 mmol at 125 mmol deoxynucleoside triphosphates (dNTPs) emerged as

the most potent inhibitor of human telomerase *in vitro*, consistent with the antitumor activities of PMEG. The PMEG-MP and PMEG itself did not show any effect on telomerase activity. The effects of PMEG on telomerase appear to be marginal compared to the inhibition of cellular DNA polymerases by PMEG-DP [IC_{50} = 2.50 ± 0.97 μM (DNA polymerase α), 1.60 ± 0.53 (DNA polymerase β) and 59.4 ± 17.6 (DNA polymerase γ) (Wolfgang et al., 2009).

In a follow-up study, the authors found that PMEG and PMEDAP were able to differently modulate telomere length in T-lymphoblastic leukemia cell lines (Hajek et al., 2010). The most striking difference concerned the CCRF-CEM and MOLT-4 cells. While in CCRF-CEM cells delayed and progressive telomere shortening was observed, MOLT-4 cells responded to the treatment by a rapid telomere elongation that could be observed as early as after 3 days of incubation and remained elevated throughout the treatment. This cell specific effect on telomere shortening was not due to direct telomerase inhibition or impairment of hTERT expression. Hajec and collaborators (Hajek et al., 2010) speculated about the mechanism of the observed telomere elongation in MOLT-4 cells. Considering that both PMEG and PMEDAP can activate and up-regulate poly (ADP-ribose)polymerase (PARP), a similar effect can be possibly anticipated on tankyrase, which is a telomeric protein possessing PARP activity. Tankyrase inhibits binding of TRF1 to telomeric DNA in vitro, where under normal conditions TRF1 prevents the access of telomerase to telomeric complex. Therefore, overexpression and/or activation of tankyrase in telomerase positive cells may induce telomere elongation without a direct effect on telomerase activity. Another possible explanation of the increase in the mean telomere length can be activation of a different telomere maintenance mechanism, termed "alternative lengthening of telomeres" (ALT), a recombination mediated process that enables survival of telomerase-negative cancer cells. It was also suggested that the factors determining the PMEG- and PMEDAP-induced telomere shortening might depend on p53 functional status (CCRF-CEM – mutated, MOLT-4 – wild-type since telomere length is connected with p53 expression and functional status and cells with mutated p53 may be more susceptible to telomere shortening induced by external stimuli (chemotherapy, irradiation, etc.). Besides, oxidative degradation of telomeres by reactive oxygen species leaking from the damaged mitochondria following treatment with PMEG and PMEDAP may affect telomere length as cells treated with these drugs were found to contain elevated levels of reactive oxygen species and telomeres have been shown to be highly susceptible to oxidative stress.

Otova and co-workers suggested that DNA-damage induced by ANPs should affect signalling pathways associated with cell proliferation, apoptosis and angiogenesis (Otova et al., 2009). They demonstrated that the antitumor efficacy of PMEG and PMEDAP in spontaneous lymphomas in rats was not only caused by inhibition of DNA synthesis but also by an effect on angiogenesis, a process stimulated by the secretion of various signalling molecules to promote neovascular formation. PMEG was found to down-regulate selected proangiogenic genes much more efficiently than PMEDAP (Otova et al., 2009).

In addition, the involvement of mitogen activated protein kinases (MAPKs) in the cytotoxicity of PME derivatives has also been reported in leukemic cell lines (Mertlikova-Kaiserova et al., 2012). MAPKs comprise a family of serine/threonine kinases that convert extracellular signals, such as stress stimuli and cytokines, into a variety of cellular processes including cell proliferation, survival, death, and differentiation. The best characterized groups of MAPKs in mammals include the extracellular signal-related kinases (ERK), c-Jun N-terminal kinase (JNK) and p38. The ERK and p38 pathways were found to be activated by PMEG and PMEDAP in leukemic cells and pretreatment with a p38 inhibitor diminished PMEG- and PMEDAP-induced apoptosis whereas inhibition of ERK, JNK or AKT (also known as protein kinase B) pathways did not (Mertlikova-Kaiserova et al., 2012).

6. Cidofovir activity against viruses not encoding for their own DNA polymerases

CDV can be given intravenously, intralesional or topically. Systemic administration of the drug requires co-administration of oral probenecid and intravenous hydration in order to prevent nephrotoxicity which is the dose-limiting clinical adverse effect of CDV. The drug is accumulated in the kidney where it reaches significantly higher concentration levels compared with other organs and tissues (Cundy et al., 1996,Cundy, 1999). The uptake of CDV across the basolateral tubular membrane is more efficient than the subsequent secretion into tubular lumen resulting in drug accumulation in renal tubules. CDV was shown to be a substrate for human and rat renal organic transport 1 (OAT1) and intravenous hydration and administration of oral probenecid [an inhibitor of OAT1 that interferes with the transporter-mediated tubular uptake of cidofovir] are used in order to prevent CDV-induced nephrotoxicity (Cihlar et al., 1999,Cihlar et al., 2001).

CDV is given mostly systemic for the management of PyV-associated diseases, although Intravesical CDV-instillation therapy for polyomavirus-associated hemorrhagic cystitis (Koskenvuo et al., 2013,Eisen et al., 2009,Mackey, 2012) and topical CDV for treatment of trichodysplasia spinulosa virus (TSPyV) (van der Meijden E. et al., 2010,Wanat et al., 2012) have been reported.

CDV has been mostly used intralesional or topically for the management of HPV-related diseases, being the therapy usually well-tolerated with minimal, if any, side effects, pointing to the selectivity of CDV for the affected tissue. In case of appearance of local side effects (presented as ulcerations at the site of the affected mucosa but not in the surrounding normal tissue), these are self-limiting and do not need cessation of treatment (Stier et al., 2013,Tjon Pian Gi et al., 2013).

Although polyoma- and papillomaviruses lack their own polymerases, off-label use of CDV, mostly in immunocompromised individuals, has proven effective in the management of diseases caused by HPV. The compound has also been used off-label for therapy of human PyV-associated illnesses with more controversial results. A puzzling situation has been why cidofovir inhibits papilloma- and polyomaviruses even though the effects of CDVpp on cellular DNA polymerization are weak compared to PMEG [inhibition constant (Ki) of CDVpp

for cellular DNA polymerase α of 51 µM *versus* 0.55 µM for PMEGpp] (Wolfgang et al., 2009,Kramata et al., 1996,Kramata et al., 1998). Another important difference between PME derivatives and CDV is the fact that CDVpp can still be incorporated during DNA elongation as CDV has a 3'-OH moiety.

6.1 *In vitro*, *in vivo* and clinical evidences for the anti-polyomavirus activity of CDV

CDV proved active against murine and primate non-human PyVs (i.e. SV40) (Andrei et al., 1997,Lebeau et al., 2007) as well as against human BKPyV and JCPyV (Topalis et al., 2011,Farasati et al., 2005,Gosert et al., 2011,Rinaldo et al., 2010) replication *in vitro*. Despite CDV shows modest in vitro activity against BKPyV, CDV is the drug most frequently used clinically to block BKPyV replication. Although the data are based solely on case reports, CDV does appear to be effective, albeit inconsistently, for the treatment of BKPyV and JCPyV infections (Kwon et al., 2013,De Luca et al., 2008,Ripellino et al., 2011,Savona et al., 2007). CDV proved also active in cases associated with productive infection of TSPyV and MCPyV in immunocompromised patients when the drug was administered topically (van der Meijden E. et al., 2010,van Boheemen et al., 2014,Wanat et al., 2012) or intravenously (Maximova et al., 2013) . CDV has been used mostly systemic for the management of BKPyV and JCPyV related diseases, although intravesical instillation of CDV has been used to manage BKPyV-associated hemorrhagic cystitis in hematopoietic stem cell transplant recipients (Koskenvuo et al., 2013,Cesaro et al., 2013,Ganguly et al., 2010).

For the management of BKPyV infections, a low dose intravenous CDV regimen of 0.25-1.0 mg/kg weekly is used empirically. The use of adjuvant low-dose CDV therapy was shown to result in prolonged graft survival and stabilized graft function in renal transplant recipients suffering from BKPyV interstitial nephritis (Kuypers et al., 2005). A recent study has demonstrated that CDV clearance and the mean estimated glomerular filtration rate in renal transplant recipients with persistent BKPyV viremia without nephropathy were linearly related irrespective of probenecid administration (Momper et al., 2013). Based on this relationship, the systemic exposure to CDV in individual patients can be predicted and may be used to evaluate exposure-response relationships to optimize CDV dosing regimen for BKPyV infection.

One may question why inconsistent results have been reported for CDV in the therapy of human PyV-associated diseases. It can be hypothesized that the pathology resulting from the relative contributions of viral replication and host response in human PyV-associated diseases may explain, at least in part, why the efficacy of CDV may vary among different patients. The diverse human PyV pathologies are the consequence of diverse viral and immunological processes that drive the disease, as reviewed by (Dalianis and Hirsch, 2013). For some human PyV pathologies such as PyVAN, HC, and PML, a reduction in viral load may be a good marker of efficacy of an antiviral drug because these pathologies are associated with high levels of viral replication. However, in cases of autoimmune or oncogenic pathology that is independent of viral replication, other markers for drug efficacy need to be developed.

The usefulness of CDV for the treatment of PML in HIV-positive patients is rather controversial. There are studies supporting a therapeutic efficacy of CDV (De Luca et al., 2000,De Luca et al., 1999) but its activity was not proven in a multicohort analysis (De Luca et al., 2008). Similarly, in HIV-negative patients some studies report efficacy (Naess et al., 2010,Viallard et al., 2007,Viallard et al., 2005) and others lack of activity (Osorio et al., 2002). If one considers that restoring the immune response in the host is one of the crucial steps in PML therapy in HIV-negative individuals and highly active antiretroviral therapy is the first treatment option for PML in HIV-positive patients, the immune status of the patient, the time of addition and dose of CDV administered may indeed have an impact on the response to treatment.

Of particular relevance in the treatment of PML is the question of the penetration of CDV across the blood-brain barrier because according to the product labelling there is no penetration of the drug into the CNS following intravenous administration.

A point that needs to be mentioned is the challenge of diagnosing PML in patients with sarcoidosis because neurosarcoidosis presents a similar pathology to that seen in PML. While neurosarcoidosis is usually treated with steroid therapy, this treatment results in enhancement of JCPyV replication in PML. Therefore, a misdiagnosis of PML may explain the lack of activity of CDV in patients previously receiving steroid therapy (Volker et al., 2007,Granot et al., 2009). Recent reports demonstrated the efficacy of CDV alone (De Raedt et al., 2008) or in combination with the anti-depressant mirtazapine (a blocker of receptors used by JCPyV to infect human glial cells) (Owczarczyk et al., 2007,Park et al., 2011) for the therapy of PML in patients with sarcoidosis that did not receive previous steroid treatment. Furthermore, combination of CDV and mirtazapine found to be helpful in the treatment of PML in HIV-negative patients (Ripellino et al., 2011).

Most predisposing risk factors for BKPyV reactivation and development of PyVAN are directly or indirectly associated with the function and activity of the immune response. Issues to be considered include: age of the patient and of the donor, viral co-infections, placement of urethral stents, the degree of HLA mismatch, episodes of acute rejection, BKPyV-specific antibody status, male sex, white ethnicity, being immunosuppressive therapy and its intensity the most important risk factor (Babel et al., 2011). As these factors might trigger or promote viral replication and increase susceptibility to PyVAN, they may affect the efficacy of adjuvant therapies, such as CDV. A comparison of the available data from case series and retrospective studies is further complicated by differences in the type of immunosuppressive therapy, patient's characteristics, CDV doses (varying from 0.25 mg/kg to 1 mg/kg), duration of treatment (3-10 weekly cycles) and use of probenecid (Kuypers, 2012).

A reduction of immunosuppression (which facilitates re-establishment of BKPyV-specific immunity) is used to prevent graft failure in many patients (Babel et al., 2011). However, this approach does not work in all individuals, raising questions about the reasons why patients respond differently following treatment with comparable protocols. Based on the pathogenesis of PyVAN, a reduction of immunosuppression can lead to a beneficial outcome only at an early stage of BKPyV infection while reduction of immunosuppressive therapy can

be damaging in patients with persistent, uncontrolled BKPyV replication and may not be considered as a therapeutic option. Thus, a reduction of immunosuppression to improve antiviral immunity appears to be more harmful than beneficial in patients with long-lasting BKPyV infection and this may also impact the effects of adjuvant therapies such as CDV.

Although supportive care has been the standard of treatment for HC during many years, several clinical studies have demonstrated successful use of CDV for BKPyV-HC after hematopoietic stem cell transplantation not only in adults but also in children (Savona et al., 2007,Cesaro et al., 2013,Gaziev et al., 2010). Important factors in the pathogenesis of HC involve severe immune suppression together with urothelial damage due to conditioning and radiation (which creates a favourable environment for viral replication and leads to an augmentation in immunological signals and antigen presentation) and the attack of virus-infected urothelial cells by donor T cells. Additional risk factors for HC include donor origin, NCCR (non-coding control region) viral mutants, treatment with anti-thymocyte globulins and type of conditioning. All these factors may influence the response to adjuvant therapies.

6.2 Polyomavirus replicative cycle: potential targets for CDV

It has been shown that CDV does not affect early steps of PyV replication such as receptor binding and entry (Bernhoff et al., 2008). Neither initial transcription nor expression of the LT-ag was impaired by CDV. However, the drug reduced intracellular BKPyV DNA replication by >90% while at equivalent concentrations a reduction of cellular DNA replication and metabolic activity of 7% and 11%, respectively, in uninfected human renal tubular cells was found. Furthermore, BKPyV infection increased cellular DNA replication to 142% and metabolic activity to 116%, respectively, which were reduced by CDV to levels of uninfected untreated cells.

Our laboratory selected SV40 mutants resistant to CDV, following growth of the virus in increasing drug-concentration in the Monkey African green kidney epithelial cell line BSC-1. This system was used because the entire lytic replicative cycle of SV40 is accomplished. CDV-resistant viruses bear mutations in the ORI and helicase domains of the LT-ag, indicating that the helicase activity required for viral DNA unwinding during replication may be affected by CDV (our unpublished data). Further research is required to prove that the helicase/ATPase activity of the LT-ag is affected by CDV and/or its metabolites.

Interference with the helicase/ATPase activity of the LT-ag may explain the activity of CDV during PyV productive infection but not against PyV-induced tumors. Liekens and collaborators reported the activity of CDV against cerebral hemangiomas induced following intraperitoneal inoculation of newborn rats with mouse PyV (Liekens et al., 1998). The drug was able to completely suppress hemangioma development even when applied 3 days following viral inoculation and resulted in 40% survival and delay in tumor-associated mortality when treatment started at the time cerebral hemangiomas were macroscopically visible (i.e. 9 days post-viral infection). Infectious virus or viral DNA were not detected in the brain of the infected animals at any time post-infection, indicating that there was not viral replication in mouse PyV-infected rats and that an antitumor effect

of CDV should be responsible for the activity of the drug in this model. A similar mode of action was postulated to explain the efficacy of CDV on the growth of hemangiosarcomas in mice originating from PyV-transformed (PV/2b/35) cells which do not produce infectious virus but express the viral T antigen (Liekens et al., 2001). CDV was also found to induce apoptosis in the hemangiosarcomas.

CDV anti-proliferative effects against PyVs can be explained by the cells infected by PyV being more sensitive to the drug because of the effects of the LT-ag on G1/S cell checkpoint deregulation. This is sustained by the higher antiproliferative effects of CDV against LT-ag transformed cells compared to the corresponding non-transformed cells (Andrei et al., 1998a).

6.3 *In vitro*, *in vivo* and clinical evidences for the anti-papillomavirus activity of CDV

The *in vitro* antiproliferative activities of CDV were first reported in 1998 (Andrei et al., 1998a) and later confirmed in several studies (Johnson and Gangemi, 1999,Johnson and Gangemi, 2003,Abdulkarim and Bourhis, 2001,Abdulkarim et al., 2002). CDV was shown not only to inhibit the growth of cervical carcinoma xenografts in athymic nude mice (Andrei et al., 1998b,Yang et al., 2010), but also to improve the pathology associated with tumor growth (De Schutter et al., 2013a). Intratumoral administration of CDV resulted in a beneficial effect on body weight gain, a reduction in splenomegaly, a partial restoration of tryptophan catabolism, and diminished the inflammatory state induced by the xenografts. The beneficial effect of CDV on the host inflammatory response was evidenced by a reduction in the number of immune cells in the spleen, histopathology of the spleen and levels of host pro-cachectic cytokines. Also, a decrease in tumor (human)-derived cytokines was measured following CDV administration. Furthermore, the positive effects of intratumoral CDV (including increased body weight gain and decreased inflammatory response) correlated with a reduction in tumor size (De Schutter et al., 2013a).

CDV is the only ANP successfully used as an antiproliferative agent in humans. Several reports have highlighted the efficacy of CDV against HPV-associated malignancies, including hypopharyngeal and esophageal (Van Cutsem et al., 1995), gingival and oral neoplasias (Collette and Zechel, 2011) as well as several anogenital neoplasias such as cervical (Snoeck et al., 2000,Van Pachterbeke et al., 2009), vulvar (Koonsaeng et al., 2001,Tristram and Fiander, 2005,Stier et al., 2013), and perianal intraepithelial neoplasias (Snoeck et al., 1995). It should be noted that in the neoplasias successfully treated with CDV, no viral productive infection is detected and only a limited number of viral genes are expressed.

Over the last years, CDV has increasingly been used as therapy for severe recurrent anogenital warts associated with the low-risk HPV6 and HPV11 types (Coremans and Snoeck, 2009,Gormley and Kovarik, 2012,Calisto and Arcangeli, 2003). The efficacy of CDV for this indication has been documented in several case reports as well as in two clinical trials [one in immunocompetent individuals (Snoeck et al., 2001) and the other one in HIV-infected patients (Matteelli et al., 2001)]. CDV has also been employed to manage recalcitrant cases

of verruca vulgaris, mosaic verruca plana, and different skin lesions caused by HPV (Stragier et al., 2002,Bonatti et al., 2007,Kralund et al., 2011,Field et al., 2009).

Importantly, following the first report on the use of CDV for the treatment of severe RRP in 1998 (Snoeck et al., 1998), the application of the drug as adjuvant therapy for severe cases of RRR has been documented by several investigators (Derkay et al., 2013,Mikolajczak et al., 2012,Ksiazek et al., 2011,Derkay and Wiatrak, 2008). Although there were some anecdotal reports documenting serious adverse reactions in RRP in off-label use of CDV (Tjon Pian Gi et al., 2012), a multicentre retrospective chart review involving 16 hospitals from 11 countries worldwide with 635 RRP patients (of whom 275 were treated with CDV) was performed. In this study, no clinical evidence was found for more long-term nephrotoxicity, neutropenia or laryngeal malignancies after intralesional administration of CDV (Tjon Pian Gi et al., 2013). In another recent study, it was concluded that CDV remains the leading option for adjuvant treatment of patients with RRP of all ages whose disease is difficult to manage with surgery alone. CDV represents an option to reduce the risks of frequent surgical debulking and airway obstruction in children and adults with recurrent or severe disease (Derkay et al., 2013). CDV is nowadays recognized as an adjuvant therapy for the management of this disease (Tjon Pian Gi et al., 2013,Graupp et al., 2013). A type specific real-time PCR to measure HPV6 and HPV11 DNA loads in patients with recurrent respiratory papillomatosis treated with CDV, indicated that the drug significantly reduced viral load following intralesional application (Mikolajczak et al., 2012). Although CDV has been reported to be ineffective in the treatment of epidermodysplasia verruciformis (a rare inherited disease characterized by widespread HPV infection of the skin) (Preiser et al., 2000), a more recent study documented its efficacy against epidermodysplasia verruciformis caused by novel HPV types (Darwich et al., 2011).

6.4 Why is CDV selective against HPV-induced hyperproliferation?

The anti-proliferative effects of CDV against HPV-induced transformation have intensively been studied the last years. The first studies showing the cytostatic activity of the drug against cervical carcinoma cells date from 1998 (Andrei et al., 1998a), where CDV and related ANPs displayed time-dependent anti-proliferative effects, in contrast to what is normally seen with chemotherapeutic drugs. HPV- and PyV-transformed cells appeared to be more sensitive to the effects of CDV due to the fact that the viral oncoproteins induce cellular proliferation making the cells more sensitive to the anti-proliferative drug effects. Thus, the activity of CDV against HPV- and PyV-transformed cells may be explained, at least in part, by an inhibitory effect of the compound on rapidly dividing cells, and the presence of the HPV or PyV genome might enhance the sensitivity of the cells to CDV. When various cell lines not containing HPV (i.e. human melanomas, lung carcinomas, colon carcinomas, breast carcinomas) were tested, CDV also showed an anti-proliferative effect (Andrei et al., 1998a).

CDV was demonstrated to induce apoptosis in cervical carcinoma cell lines and to arrest the cells in the S-phase of the cell cycle with increased levels of the tumor suppressor proteins p53 and pRb and of the cyclin-

768 dependent kinase inhibitor p21/WAF-1. Thus, CDV was able to restore the function of p53 and pRb, which are
769 neutralized by the oncoproteins E6 and E7, respectively, in HPV-transformed cells (Andrei et al., 2000). Induction
770 of apoptosis by CDV was confirmed later in several tumor models, including human cancer xenografts in athymic
771 nude mice (Yang et al., 2010,Abdulkarim et al., 2002).

772 CDV proved to reduce E6 and E7 expression in the HPV-18 positive cervical carcinoma ME-180 cells and in the
773 HEP-2 cells (originally believed to be derived from a head and neck squamous cell carcinoma but later turned out
774 to be HeLa cells) at the transcriptional level with subsequent reactivation of p53 and pRb (Abdulkarim et al.,
775 2002). In a model of stromal-derived factor 1 (SDF-1α)-stimulated invasiveness of HPV-positive cells, CDV had
776 anti-metastatic action which was mediated by inhibition of E6/E7, CXCR4 and Rho/ROCK signalling (Amine et al.,
777 2009).

778 Donne and co-workers tested the effects of CDV on the non-HPV cervical carcinoma cell line C33A compared
779 to two derived cell lines, i.e. the C33AT6E6 cells (stable transfected with the low risk HPV6 E6) and the
780 C33AT16E6 cells (stable transfected with the high-risk HPV16 E6). The authors found that CDV treatment had a
781 marked growth-inhibitory effect on high-risk E6 expressing C33AT16E6 cells, supporting the use of CDV for
782 treatment of high-risk HPV-associated diseases. However, unlike high-risk E6, expression of low-risk HPV E6 in
783 C33A cells did not augment the sensitivity of these cells to CDV. The authors conclude from their studies that
784 CDV may have little selectivity for low-risk HPV related diseases. However, they based their conclusion only on
785 the expression of one of the viral oncoproteins neglecting the fact that low-risk HPV lesions are due to HPV-
786 induced hyperproliferation resulting from productive HPV infection. On the other hand, Donne's experiments
787 presumably used newly transfected E6 and E7 expression vectors that had not replicated in the presence of CDV
788 and therefore would not have incorporated CDV to block transcription. On the other hand, they tested the
789 effects of CDV on expression of HPV6b and HPV16 E6 mRNA levels in a system that over-expresses these viral
790 proteins. Also, they used the cervical carcinoma HPV-negative cell line C33A which is also sensitive to the
791 antiproliferative effects of CDV. In contrast to previous results, they found increased HPV E6 RNA levels in C33A
792 cells that over-expressed HPV6b or HPV16 E6 and no selectivity of CDV for HPV-positive cells (Donne et al.,
793 2009,Donne et al., 2007).

794 A crucial difference between normal keratinocytes, benignly HPV-infected cells, and immortalized or
795 transformed cells is the frequency as well as level of induction of host enzymes associated with energy
796 metabolism and with S-phase entry and cell cycle progression to G2 phase, driven by the PyV LT-ag or the PV E7
797 protein. Notably, the DNA viruses strongly up-regulate glycolysis including kinases such as pyruvate kinase. It can
798 be hypothesized that phosphorylation of CDV and other ANPs might be selectively activated in this productive or
799 transformed environment compared to more quiescent normal cells. Accordingly, to explain the selectivity of
800 CDV for HPV-positive cells, Johnson and Gangemi (Johnson and Gangemi, 1999) claimed that CDV could be
801 differentially metabolized in HPV-positive cells and normal keratinocytes. Following 8 and 16 hours incubation,

CDV was found to predominantly accumulate in the form of CDVp-choline (considered the intracellular depot form of CDV) in human primary keratinocytes (PHKs) while in HPV16-transformed keratinocytes, CDVpp was the most abundant anabolic product with little CDVp-choline having formed.

Recently, we reported that following 72 h incubation with CDV, CDVp-choline appeared to be the most abundant metabolite while the monophosphate form was the least abundant one in PHKs as well as in HPV-positive and HPV-negative tumor cells (De Schutter et al., 2013c). Importantly, no significant differences in the levels of the active metabolite CDVpp, CDVp-choline or CDV were observed between PHKs and HPV-positive tumor cells. However, lower CDVp levels were measured in PHKs compared to HPV-positive cells following 72 h incubation. Notably, lower concentrations of CDV and of all metabolites were observed in the spontaneously transformed keratinocyte cell line HaCaT that lack HPV sequences, compared to either HPV-positive cells or PHKs, suggesting that HaCaT cells have a different uptake and/or efflux of CDV, rather than differences in drug metabolism.

To reveal the molecular mechanisms underlying the selectivity of CDV for tumor cells, in particular for HPV-positive carcinoma cells, our research team evaluated gene expression changes following CDV treatment of different cell types [including two HPV-positive cervical carcinoma cell lines (SiHa, HPV16+ and HeLa HPV18+), an HPV-immortalized keratinocyte cell line (HaCaT), and PHKs (De Schutter et al., 2013c). In addition, drug incorporation into genomic DNA was analysed in the four cell types. An exhaustive and thorough analysis of the microarray data highlighted distinct responses to CDV exposure in PHKs compared to HPV-positive cervical carcinoma cells, on the one hand, and to HPV-immortalized keratinocytes, on the other hand. Our data indicated that the selectivity of CDV for HPV-transformed cells is based on differences in response to DNA damage, replication rate and CDV incorporation into cellular DNA between immortalized cells and normal cells, rather than on a specific effect of CDV on expression of the viral oncoproteins (De Schutter et al., 2013c). Normal cells possess an arsenal of repair pathways and cell cycle checkpoints to detect and repair DNA damage unlike transformed cells that have a significantly reduced set of DNA repair pathways for survival **(Figure 3A)**.

The presence of E6 and E7 appears to indirectly contribute to the efficacy and selectivity of CDV because the role of E7 interactions with host cell cycle control proteins is (a) to reactivate cell cycle (notably S-phase) in differentiated cells that had withdrawn from the cell cycle, and (b) to trigger unscheduled DNA synthesis. As a consequence, E7 quickly leads to the stabilization of p53 and hence the need for E6:E6AP to neutralize p53 or lead to its ubiquitinylation and proteasome-mediated turnover.

The selective mechanism of action of CDV as antiproliferative agent could be inferred by analyzing the specific signatures identified in CDV-exposed PHKs that were not found in tumor cells, including cell cycle regulation and activation of DNA-double strand breaks (DSBs) repair mechanisms (i.e. 'ATM Signalling' and 'Double-Strand Break Repair by Homologous Recombination') **(Figure 3B)**. These findings suggest that CDV can

generate double-strand DNA breaks that cannot be repaired by tumor cells but well by normal cells (De Schutter et al., 2013c).

Furthermore, when we compared the efficiency of CDV incorporation into genomic DNA in the different cell types, higher amounts of CDV were incorporated in the genomic DNA of transformed epithelial cells compared to PHKs, despite the fact that the levels of intracellular CDV metabolites were not significantly different among the cell types investigated. Recently, these findings were confirmed by P**.** Hadaczek and co-workers who also found that CDV is incorporated into cellular DNA activating DNA damage response pathways due to increased DNA breaks that prompt elevated tumor cell apoptotic response in glioblastoma cells (Hadaczek et al., 2013).

Besides differences in cell cycle regulation and DNA repair pathways, our gene expression profiling analysis also allowed the identification of other pathways and functions that were induced or repressed following exposure to CDV differently in PHKs compared to HPV-positive and/or HPV-negative cells (De Schutter et al., 2013c). For instance, Rho GTPase pathways and the acute phase response pathway were solely activated in immortalized cells while normal keratinocytes showed the activation of several metabolic pathways **(Figure 4)**. Therefore, besides induction of double-strand DNA breaks, CDV showed a differential effect on specific pathways in normal cells compared to transformed cells that may contribute to the activity and selectivity of the drug for tumor cells.

Furthermore, *in vitro* acquisition of resistance to CDV in SiHa cells was found to implicate a variety of cellular functions and pathways linked to cell death, cell growth and differentiation, cellular movement, metabolism, tissue development as well as inflammatory response (De Schutter et al., 2013b). Notably, SiHa cells selected for resistance to CDV presented a reduced growth *in vitro* and in the mouse xenograft model inducing a reduced inflammation, as measured by a reduced production of mice- and human-derived cytokines, diminished effect on chemical and haematological blood parameters, lower number of cells in the spleen and lesser splenomegaly compared to parental cells. Interestingly, when parental and CDV-resistant cells produced an equivalent size of xenografts (i.e. 3 and 5 weeks post cell-inoculation), the amount of neutrophils, macrophages, NK cells and inflammatory cytokines was significantly higher in the animals inoculated with the parental cells compared to those that received the CDV-resistant cells.

7. Extension of the clinical use of CDV for the treatment of tumors not induced by oncogenic viruses

Our data obtained by whole genome gene expression profiling of normal *versus* immortalized cells exposed to CDV supports the use of CDV for the treatment of non-viral induced neoplasias. Furthermore, a few reports sustain this hypothesis. For instance, CDV proved effective in reducing the growth of melanoma B16 in an experimental model in mice (Redondo et al., 2000). The most effective treatment in this model was subcutaneous administration of 67 mg/kg on alternative days three times weekly that resulted in 90% inhibition of tumor growth.

When CDV antiproliferative effects were evaluated against a series of nine HPV-negative cells, the 50% cytostatic concentrations of the drug following 7 days of incubation varied between 1.4 µg/ml (for the cervical carcinoma cell line C33A) and 43 µg/ml (for the breast carcinoma cell line BT-20) compared to 0.7 to 2.0 µg/ml for four different HPV-positive cell lines [SiHa and CaSki (HPV-16), HeLa (HPV-18) and CK-1 (HPV-33) (Andrei et al., 1998a). When ODE-CDV was compared to CDV, ODE-CDV proved more potent than the parent compound against the HPV-positive cell carcinoma cell lines HeLa, CaSki, Me-180 (HPV-68) and the C33A cervical carcinoma cells lacking HPV (Hostetler et al., 2006).

Liekens et al have demonstrated the inhibitory effects of CDV on the development of virus-independent vascular tumors originated by basic fibroblast growth factor (FGF2)-overexpressing endothelial cells (FGF2-T-MAE). The *in vivo* antitumor efficacy of CDV was attributed to specific induction of apoptosis in this model (Liekens et al., 2007). In addition, CDV treatment of FGF2-T-MAE cells resulted in a pronounced up-regulation of the tumor suppressor protein p53. However, the expression of Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) proteins remained unchanged, and CDV did not induce the release of cytochrome c from the mitochondria. Therefore CDV appeared to inhibit the growth of FGF2-T-MAE cells via inhibition of FGF2 expression and signalling (Liekens et al., 2007).

Recently, it was shown that CDV possesses potent antineoplastic activity against both HCMV positive and negative glioblastomas (Hadaczek et al., 2013). While this activity was associated with inhibition of HCMV expression and with activation of cellular apoptosis in HCMV-positive glioblastomas, CDV was also demonstrated to induce cell death in the absence of HCMV. CDV incorporated into tumor cell DNA promoting double-strand DNA breaks and apoptosis.

8. Can the antitumor activity of CDV be synergized?

Considering the mode of cell killing by CDV, combination of CDV and radiation therapy can be considered as a promising and feasible strategy to improve treatment outcomes for different tumor types, of both viral and non-viral origin.

By the addition of further DNA damage, such as irradiation therapy, it can be hypothesized that cellular apoptotic response to CDV would increase. Indeed, combining CDV with irradiation both *in vitro* and in engrafted nude mice resulted in a marked radio-sensitization in HPV-positive cells, which was not observed in HPV-uninfected cells (Abdulkarim et al., 2002). The synergistic effect of CDV and radiation in HNSCC cells was associated with p53 accumulation. It has also been shown that the combination of CDV and radiation had a potent anti-angiogenic effect, inducing inhibition of E6 expression, restoration of p53, and reduction of the pro-angiogenic phenotype of HPV18 positive cells associated with VEGF (vascular endothelial growth factor) inhibition (Amine et al., 2006). CDV also enhanced the radiation-induced apoptosis in EBV-positive cells and in EBV-related cancer xenografts (Abdulkarim et al., 2003). CDV induced a downregulation of the EBV oncoprotein

LMP1 associated with a decrease in expression of the anti-apoptotic Bcl-2 protein and an increase of the pro-apoptotic Bax protein in Raji (Burkitt lymphoma) and C15 (nasopharyngeal carcinoma) cells (Abdulkarim et al., 2003).

The antitumor effect of CDV was also evaluated in combination with radiation therapy against glioblastoma (Hadaczek et al., 2013). *In vitro*, a dramatic increase (over 21-fold) of phosphorylated H2AX, an indicator of DNA damage/instability, after exposure to both CDV and ionizing radiation was observed. Furthermore, this combination resulted in reduced tumor growth in a model of human glioblastoma-derived intracranial xenografts in mice leading to increased animal survival.

On the other hand, the combination of cidofovir with chemotherapeutics presenting a different mode of antitumor action may be expected to result in synergistic antitumor activity. In line with this assumption, Deberne and colleagues investigated the combination of cidofovir with the anti-epidermal growth factor receptor monoclonal antibody cetuximab *in vitro* (using a clonogenic survival assay, cell cycle analysis, and phospho-H2AX levels) and *in vivo* (using xenograft models) (Deberne et al., 2013). This combination was assessed considering the cross-talk between epidermal growth factor receptor and HPV that is implicated in tumor progression. The CDV-cetuximab combination inhibited the growth of the different cell lines tested, including HPV-positive (HeLa and Me 180) and HPV-negative (C33A, H460 and A549) cells, with synergistic activity on HPV-positive but not on HPV-negative cells. The CDV-cetuximab combination also delayed tumor growth of HPV-positive tumors *in vivo* but no efficacy was reported on HPV-negative C33A xenografts. The combination induced S-phase arrest associated with enhanced levels of double-strand breaks (as measured by phosphorylation of H2AX) in HeLa and Me 180 cells.

9. Conclusions and Perspectives

The research performed in the last years has provided a better understanding on the mechanisms of antitumor efficacy of ANPs. Although comparative studies between CDV and ANPs of the PME series (such as PMEG) are missing, their action on cellular DNA polymerization appeared to be different, PMEG having a higher affinity for cellular DNA polymerases than CDV. An important difference between both drugs is the ability of PMEG to cause chain termination of viral DNA synthesis in contrast to CDV that can be incorporated. Although both PMEG and CDV can cause DNA damage, they may differ in the type of damage induced. In the case of CDV, it appeared that the drug is able to induce double-stranded DNA damage and that only normal cells are capable of activating a DNA damage response and repair the damage via homologous recombination (considered as a very faithful mechanism of DNA repair). On the other hand, it appears that CDV is able to trigger several signalling pathways in tumor cells, both HPV-positive and HPV-negative cells, such as Rho GTPase signalling and acute phase response that may also contribute to its antitumor efficacy and selectivity.

There is an unmet need for effective anti-HPV treatments for existing infections and for patients that do not receive the prophylactic vaccination. Also, no FDA-approved treatments exist to manage human PyV infections. The use of cidofovir derivatives such as CMX001 (with substantially improved oral bioavailability and reduced toxicity compared to CDV) and HPMP-5-azaC (with in vitro and in vivo antiproliferative effects equivalent as those described for CDV) deserve further evaluation. Also, the use of formulations of CDV should be envisaged in order to use lower drug levels and enhance efficacy. A recent study has shown that formulation of CDV improved the anti-papillomavirus activity of topical CDV treatments in the CRPV/rabbit model (Christensen et al., 2014).

Importantly, CDV was suggested to affect the LT-ag of PyV, indicating that the helicase activity associated with the LT-ag may be the target of CDV. Although there is no overall homology among the PyV and PV genomes, the helicase motif of PV E1 protein, a domain stretching about 230 amino acids, has some sequence similarity with the SV40 LT-ag (de Villiers et al., 2004). Furthermore, a comparison of the active s ite from SV40 LT-ag and HPV E1 proteins shows high similarities **(Figure 5)**. The lysine finger is conserved in the LT-ag and the HPV E1 proteins and, in addition, a number of aspartates, asparagines and threonines are conserved in the active site of both types of proteins. Structural similarities between the LT-ag and the BPV E1 protein have also been described (Topalis et al., 2013). This opens new perspectives in the understanding of the mechanism of inhibition by CDV during productive PyV and HPV replication as the E1 HPV protein also has helicase activity. Various validated systems for testing the components of HPV E1 helicase and viral DNA replication using transient transfection of E1 and E2 expression plasmids or using purified enzymes in vitro have been reported (Liu et al., 1995,Kuo et al., 1994,Fradet-Turcotte et al., 2010).

Further research is also needed in understanding the effects of CDV on the productive replicative cycle of low-risks HPVs and the organotypic epithelial raft cultures appear to be the ideal system to perform these investigations as they reproduce epithelial differentiation in an ex vivo system. A fully productive 3-dimensional tissue culture system for production of high yields of infectious HPV-18 virions was first described in 2009, with multiple published applications since then (Wang et al., 2009).

This system appears to be also more appropriate to analyze drug-metabolism because nucleoside metabolism in cell monolayer cultures (especially with immortalized and transformed cells) are considerably abnormal compared to 3-dimensional tissues, where most cells are quiescent. Moreover, uptake of small molecules is substantially altered in rapidly dividing monolayer cells that do not have cell-cell junctions.

Nucleotide synthetic pathways have exquisitely coordinated balancing of de novo production of the ribonucleoside and the deoxyribonucleoside triphosphates, and these regulatory responses are also heavily influenced by salvage of nucleosides from broken down RNA and DNA or from the general circulation. Exogenous agents such as inhibitors of these synthetic or salvage pathways (eg. hydroxyurea, methotrexate) or from nucleoside analogs (eg. 5-FU) can substantially alter this balancing network. Whether CDV or other ANP's

have an impact on the normal distribution of ribo- and deoxyribo-nucleosides and their phosphorylated derivatives should be investigated. How CDV and other ANPs impact ribonucleoside diphosphate reductase, the main source of deoxynucleotide synthesis in virally infected cells should be considered, as well as the consequences of cell growth in the presence of CDV with respect to ribosomal RNA transcription and processing.

One of the major findings regarding CDV-antitumor activities points to the potential use of the drug in the therapy of non-viral induced tumors such as glioblastomas. Also, further research will be necessary to elucidate the effects of CDV in several signalling pathways compared to PME derivatives and other chemotherapeutics in order to highlight (dis)similarities and understand their mechanisms of action.

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Legends to the figures

Figure 1. Intracellular metabolism of CDV and mechanism of action against viruses encoding for their own DNA polymerases. Once inside the cells, CDV needs to be activated by cellular enzymes (Ho et al., 1992). UMP-CMP kinase catalyses the conversion of CDV to CDV-monophosphate (CDVp), which is then further phosphorylated to the active form, CDV-diphosphate (CDVpp) by nucleoside 5'-diphosphate kinase, pyruvate kinase or creatine kinase. CDVpp can be used by the choline phosphate cytidyltransferase to form the CDVp-choline according to the reaction : CDVpp + choline phosphate → CDVp -choline + pyrophosphate (Cihlar and Chen, 1996). Choline-phosphate cytidylyltransferase (EC 2.7.7.15) is an enzyme that catalyzes the chemical reaction CTP + choline phosphate ⇆ diphosphate + CDP-choline where the two substrates of this enzyme are CTP and choline phosphate, and the two products are diphosphate and CDP-choline. Choline phosphate cytidyltransferase is responsible for regulating phosphatidylcholine content in membranes. CDVp-choline is considered to serve as an intracellular reservoir for the mono- and diphosphate derivatives of CDV. The active form of CDV (i.e. CDVpp) interacts with the viral DNA polymerase as either competitive inhibitor [with respect to the natural substrates (i.e. dCTP)] or alternative substrate (thus leading to incorporation into DNA). CDV has a hydroxyl function in the acyclic side chain that would allow further chain elongation. For human CMV, chain termination occurs when two consecutive CDVpp are incorporated in the growing DNA chain.

Figure 2. Scaffold of PME and HPMP derivatives. Chemical structures of PME and HPMP derivatives that are known for their antiproliferative activities. Chemsketch has been used to draw the structures of the different drugs.

Figure 3. (A) Differential response of normal cells and cancer cells to DNA damage. Adapted from (Khalil et al., 2012). Lines with arrow head indicate activation, while lines with bar head indicate inhibition. In response to DNA damage, normal cells activate the DNA damage response pathway which will cause G1/S arrest via the p53 pathway and G2/M arrest via checkpoint kinases Chk1 and Chk2 pathway. In normal cells, cell cycle arrest by either of these pathways allows time for DNA repair resulting in the prevention of genomic instability. In contrast, cancer cells have a dysfunctional G1/S checkpoint because of loss of p53 function. In addition, most of the tumor cells present alterations in the ATM pathway leading to disruption of the G2/M checkpoint. As a result, cancer cells will accumulate irreparable lesions resulting in the activation of apoptosis. **(B) Differential response of normal cells and tumor cells to CDV.** According to the microarray data reported by (De Schutter et al., 2013c), primary human keratinocytes (PHKs) activate cell cycle regulation mechanisms that allow DNA repair by means of homologous recombination (HR), leading to genomic stability and cell survival. Complete lines with

arrow heads indicate activation while dashed lines indicate alternative repair pathways that were not identified in the microarray data. In contrast to normal cells, the immortalized keratinocyte cell line HaCaT and the cervical carcinoma cell lines SiHa (HPV16) and HeLa (HPV18) are unable to repair DNA damage resulting in genomic instability and induction of apoptosis. The most relevant genes related to cell cycle regulation and/or DNA damage repair pathways found upregulated following CDV exposure of PHKs are shown in red.

Figure 4. Differential response to CDV treatment of HPV-positive and HPV-negative immortalized keratinocytes versus primary keratinocytes based on a whole genome gene expression profile reported by (De Schutter et al., 2013c). Activation of several metabolic, cell cycle regulation and DNA repair pathways were solely activated in normal keratinocytes while immortalized cells failed to induce DNA repair mechanisms following exposure to CDV. Importantly, two pathways (i.e. 'activation of Rho GTPase signaling' and 'acute phase response signaling') were exclusively identified in CDV-treated immortalized cells.

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Table 1. Clinical uses of CDV (as an antiviral and antiproliferative agent) either approved [by the Food and Drug Administration (FDA)] or supported by clinical data (De Clercq, 2003,Snoeck and De Clercq, 2002,De Clercq, 2006,De Clercq, 2011).

Route of administration	Clinical indication
Systemic (intravenous)	• HCMV retinitis in AIDS patients (approved). • HSV-1, HSV-2 and VZV infections (particularly those that are resistant to acyclovir and/or foscavir). • HCMV infections, mainly those resistant to ganciclovir due to mutations in the UL97 gene. • EBV, HHV-6, HHV-7 and HHV-8 (Kaposi's sarcoma associated herpesvirus) infections. • Polyomavirus infections due to JCPyV [progressive multifocal leukoencephalopathy (PML)] and polyoma BKPyV [hemorrhagic cystitis]. • Systemic adenovirus infections.
Systemic (intravenous) or topical (gel/cream)	• Molluscum contagiosum, orf and other poxvirus infections such as monkeypox and smallpox. • Complications of smallpox vaccine (vaccinia).
Topical (gel/cream)	• Mucocutaneous HSV-1 or HSV-2 infections (particularly those resistant to acyclovir and/or foscavir.
Topical (eyedrops)	• Keratoconjunctivitis due to HSV or adenovirus.
Topical (intravitreal)	• HCMV retinitis.
Topical (gel/cream), intralesional injection, infrequently systemic administration)	• Human papillomavirus-associated lesions: - recurrent laryngeal papillomatosis - anogenital warts - common warts - cervical/vulvar/anal/penile intraepithelial neoplasia

Herpes simplex 1 (HSV-1) and 2 (HSV-2), human cytomegalovirus (HCMV), varicella-zoster virus (VZV), human herpesvirus 6 (HHV-6), 7 (HHV-7), 8 (HHV-8). Foscavir*:* foscarnet sodium injection.

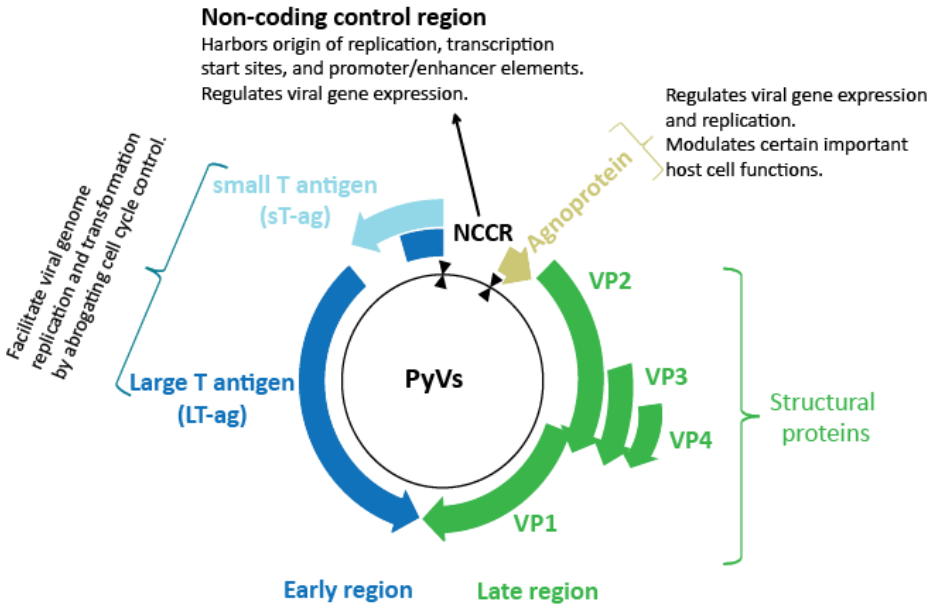
Table 2. Major similarities and differences between polyoma- and papillomaviruses.

	Polyomavirus	Papillomaviruses
Genome	• Double-stranded • Circular • DNA • ~5 kbp • Uses overlapping genes and both strands of DNA to pack all 6 genes into 5 kbp	• Double-stranded • Circular • DNA • ~8 kbp • Uses overlapping genes and one strand of DNA to pack all 7 genes into 8 kp
Morphology	• Non-enveloped • ~ 45 nm diameter • Icosahedral, T=7 • 3 capsid proteins (VP1, VP2, VP3)	• Non-enveloped • ~ 52-55 nm diameter • Icosahedral, T=7 • 2 capsid proteins (L1, L2)
Early proteins	• LT-ag • sT-ag • mT-ag (in murine PyV) • ALTO (in TSPyV)	• E1: viral replication • E2: viral replication and transcription • E4: desestabilization of cytokeratin network • E5: alteration of growth factor signaling • E6: oncoprotein • E7: oncoprotein
DNA Replication	• LT-ag is the multifunctional initiator protein • The C-terminal domain of LT-ag have ATPase/helicase activity and is sufficient for oligomeriation into hexamers • The central part of LT-ag contains an origin-binding domain (OBD) which recognizes specific sequences in the origin • The LT-ag OBD can bind with high-affinity to its target binding site as a monomer • Specific N-terminal domain of LT-ag that contains a unique J-domain, a monopartite nuclear localization signal (NLS) and several phosphorylation sites for different kinases that modulate either the nuclear import of LT-ag or its assembly into a double hexamer at the origin	• E1 is the multifunctional initiator protein but also requires E2 to initiate viral replication in vivo • E1 requires interaction with E2 to oligomerize into hexamers • Replication begins with the recruitment of E1 by E2 to the Ori and requires an essential protein-protein interaction between the E2 transactivating domain (i.e. TAD) and the E1 helicase domain • The E1 OBD needs to dimerize to bind with high-affinity to its target binding site • Specific N-terminal domain of E1 that contains a bi-partite NLS, a Crm1-dependent nuclear export signal (NES), and a binding site for cyclin A/E-cdk2 and several phosphorylation sites for different kinases that modulate either the nuclear import of LT-ag or its assembly into a double hexamer at the origin
Interaction with tumor suppressor proteins	• The LT-ag binds to both Rb family proteins and p53.	• E6 binds to p53 and other pro-apoptotic proteins and E7 binds to Rb family proteins

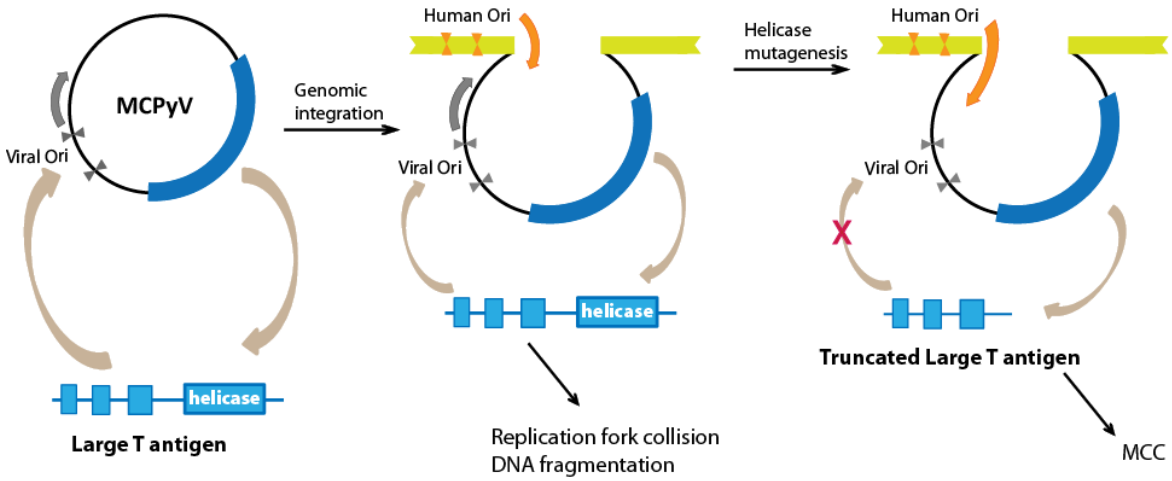
Table 3. Comparison of the ATP binding site from SV40 LT-ag and HVP-16/18/6 E1. The ATP binding site is formed from residues belonging to two adjacent monomers (*cis*- and *trans*-domains). The P-loop (sequence **G**$(X)_4$**GK**T/S) is a motif present in all the proteins harboring an ATP binding site.

[2]	Polyomavirus	High Risk HPV		Low Risk HPV
ATP binding domain	**SV40 LT-ag**	**HPV-16 E1**	**HPV-18 E1**	**HPV-6 E1**
***Trans*-domain**	K418-K419 R498, D502, R540	K469-K470 R537, D541, R581	K476-K477 R544, D548, R589	K470-471 R538, D542, R582
***Cis*-domain**	D474, N529	D523, N567	D530, N574	D524, N568
P-loop	426-**G**PIDS**GK**-432	477-**G**AANT**GK**-483	484-**G**PANT**GK**-490	478-GPPDTGK-484

Box 1.

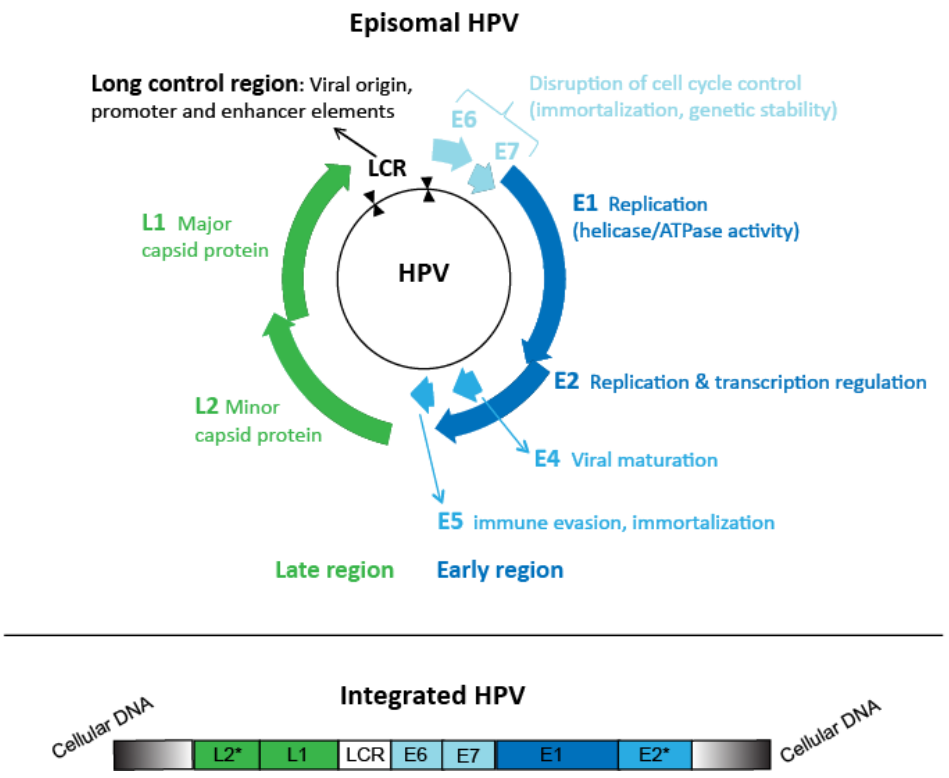


Polyomavirus (PyVs) genome organization. The PyV genome is functionally divided into coding (early and late) and non-coding regions. In all PyVs, the early coding region encodes for the Large T antigen (LT-ag) and small T antigen (sT-ag) while the late region encodes for the viral structural proteins and the non-structural agnoprotein, which is exclusively found in BKPyV and JCPyV (Khalili et al., 2005). Agnoproteins contribute to viral propagation at various stages in the replication cycle, including transcription, translation, processing of late viral proteins, assembly of virions, and viral propagation. The non-coding control region (NCCR) contains the viral promoters and origin of replication. The late region encodes the viral capsid proteins VP1-3 and the recently discovered VP4 in SV40. VP1 forms 72-pentameric capsomers, each capsomer containing a single copy of the minor structural protein VP2 or VP3 in its central cavity. VP4 is expressed during the late stages of SV40 replication and is not found in the virion. SV40 appears to initiate cell lysis by expression of VP4 which acts as a viropin that form pores resulting in perforation of cellular membranes for virus release (Raghava et al., 2011,Raghava et al., 2013). The JCPyV agnorpotein was also shown to act as a viropin (Suzuki et al., 2010).



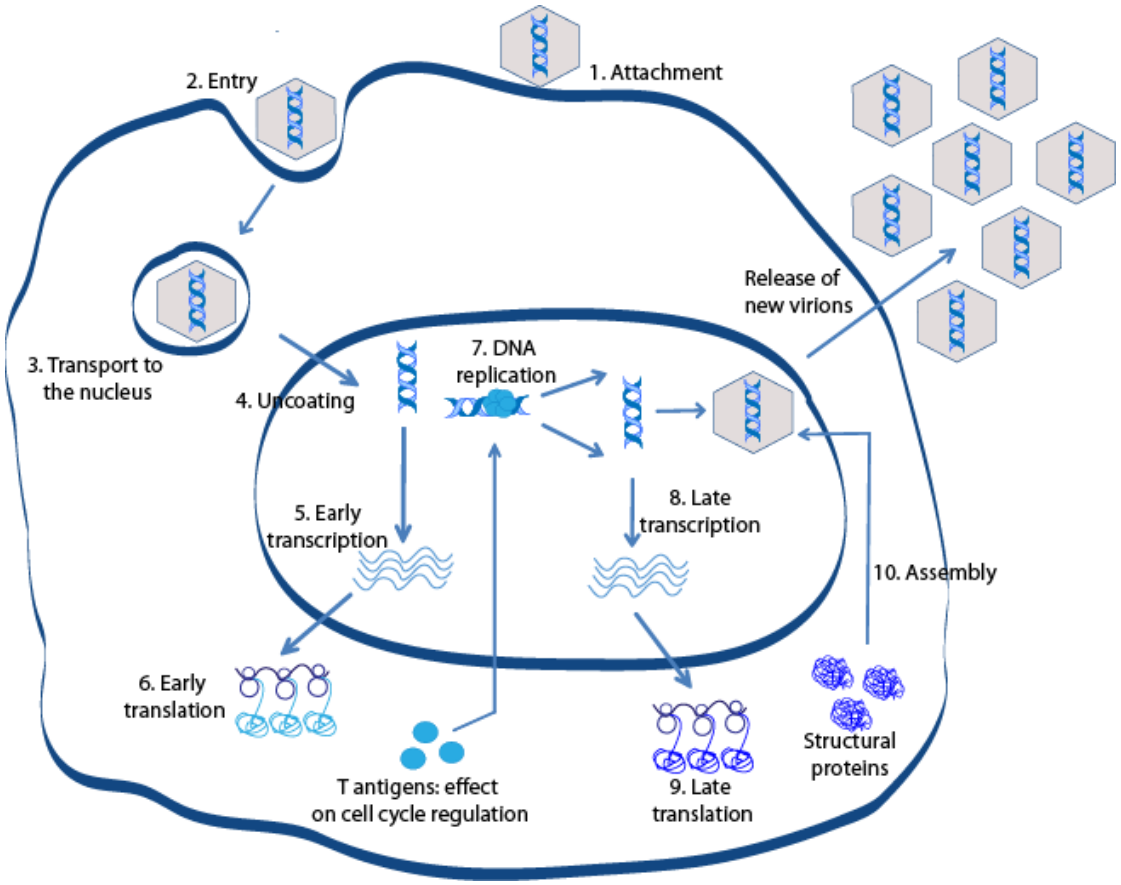
Merkel cell polyomavirus (MCPyV) integration in Merkel cell carcinoma (MCC), adapted from (Moore and Chang, 2010)**.** In case a rare integration mutation into the host cell genome occurs, the MCV T antigen can activate independent DNA replication from the integrated viral origin, which would result in replication fork collision and DNA fragmentation. Hence, a second mutation in the LT-ag that eliminates its replication functions upon integration is required for tumorigenesis. All MCC-derived LT-ag sequences analyzed so far present premature stop codons or deletions leading to abrogation of the helicase domain. These mutations are specific for MCPyV in MCC and are absent in viruses derived from non-tumor tissues. The truncation mutations eliminate LT-ag helicase activity but retain the LXCXE–retinoblastoma protein-binding motif as well as other N-terminal motifs of the LT-ag. Thus, the integrated virus in MCC retains the ability to regulate the host cell cycle and inhibit the

Box 2



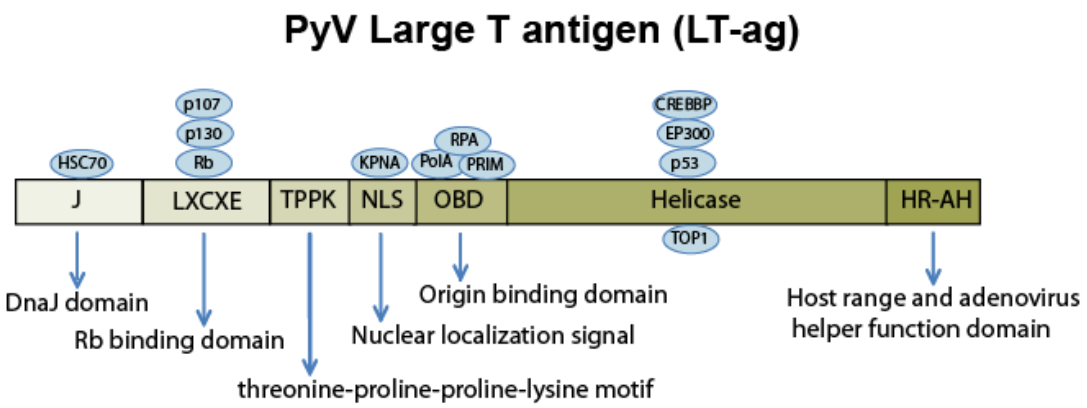
Schematic presentation of the closed circular double-stranded human papillomavirus (HPV) DNA and its integration into host-cell DNA. Adapted from (Stanley et al., 2007). In the circular HPV genome are shown the non-coding region (i.e. long control region) and the open reading frames (ORFs) encoding the early and late viral proteins. During the process of oncogenesis, the viral genome becomes integrated into host cellular DNA. The circular HPV DNA is usually opened within the E2 ORF, disrupting its continuity. Part of E2, E4, E5 and L2 are regularly deleted after integration (partial genes are represented by an asterisk).

Box 3

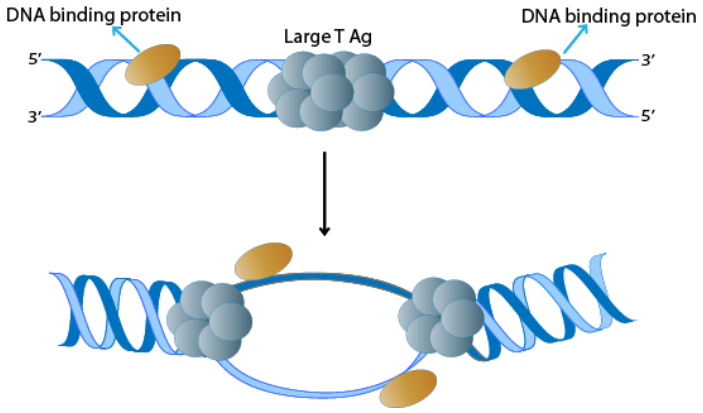


Scheme of the polyomavirus replicative cycle. To gain entry into the cells, viral capsid proteins interact directly with the receptor molecules. The PyV capsids consist of 72 pentamers of the major capsid protein viral protein 1 (VP1), which is responsible for antigenicity and receptor specificity. PyVs are internalized by the interaction of VP1 with specific cellular receptors. Gangliosides [glycosphingolipids (ceramide and oligosaccharide) with one or more sialic acids (n-acetylneuraminic acid, NANA) linked on the sugar chain] are used as receptors for most of the well-characterized polyomaviruses. For instance, gangliosides GD1a and GT1b are recognized as receptors for murine PyVs and GM1 for SV40 (Ashok and Atwood, 2006). In the case of BKPyV, it was shown that the virus uses specific gangliosides that all contain a common α2,8-disialic acid motif to infect the cells (Neu et al., 2013). Lactoseries tetrasaccharide c (LSTc) which terminates in α2,6-linked sialic acid was identified as the specific receptor for JCPyV and the presence of α2,6-linked sialic acid correlates with JCPyV cell and tissue infection, including B lymphocytes, kidney, and the glial cells astrocytes and oligodendrocytes (Hirsch et al., 2013,Maginnis et al., 2013). PyVs enter into the host cell via a caveolae-mediated endocytic pathway (Tsai and Qian, 2010). In contrast to most PyVs, JCPyV internalization relies on clathrin-dependent receptor-mediated endocytosis and is then sorted to caveosomes. Trafficking through the endoplasmatic reticulum (ER) appears to be a necessary step for all PyVs (Bennett et al., 2012). The presence of chaperons, disulfite isomerases and reductases in the ER may facilitate the capsid uncoating process. The viral genome, still associated with some viral proteins, is imported into the nucleus via importin recognition of a nuclear localization signal present in VP3. In the nucleus, early genes are expressed by the host transcriptional machinery (Fanning et al., 2009,Hirsch et al., 2013). After translation of the early proteins, the LT-ag initiates DNA replication of the viral genome that is carried out by cellular DNA polymerases. The shift to transcription of late genes is not completely elucidated but involves transcriptional activation of the late and repression of the early promoters by LT-ag. The constitutive viral capsid proteins are imported to the nucleus where encapsidation of the viral genomes occur followed by release of new virions.

Box 4

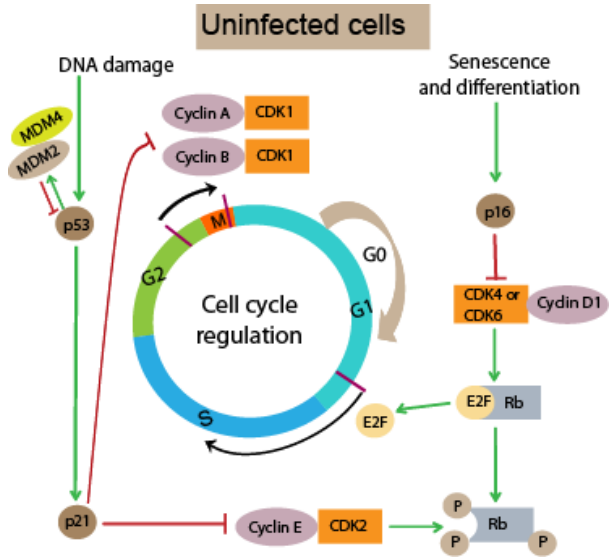


Functional domains of polyomavirus (PyV) Large T antigen (LT-ag). Adapted from (DeCaprio and Garcea, 2013). The N terminus of LT-ag contains a DnaJ domain (or J domain), necessary for efficient viral DNA replication, recruits heat shock cognate protein (HSC70) homologues. Besides the DnaJ domain, all LT-ag's contain a LXCXE motif that binds directly to the tumour suppressor retinoblastoma protein family of proteins (i.e. pRb, p130 and p107). The DnaJ domain and the LXCXE motif cooperate to disrupt the interaction of Rb and the E2F family of transcription factors in order to promote cell cycle progression. The LT-ag of all PyVs also contain a threonine-proline-proline-lysine (TPPK) motif, a nuclear localization signal (NLS), a DNA-binding domain (DBD) and a helicase domain. Phosphorylation of the threonine residue in the TPPK motif is required for LT-ag-mediated viral DNA replication. The DBD and helicase domains are required for viral replication and recruit cellular DNA replication factors [DNA polymerase-α catalytic subunit (POLA), the replication protein A complex (RPA) and the DNA primase complex (PRIM) for DBD and EP300, CREBBP, p53 and DNA topoisomerase (TOP1) for the helicase domain]. The outside surface of the helicase domain of PyVs binds to the tumor suppressor protein p53, blocking p53-dependent gene expression in response to DNA damage signals. After the helicase domain, the C-terminal region of JCPyV and BKPyV contains a C-terminal region that bears some homology with the SV40 C-terminal domain. This region of homology includes threonine-701 in SV40 that when phosphorylated competes with phosphorylated cyclin E and MYC, increasing their levels and in this way contributing to cellular growth and proliferation. The C-terminal region of SV40 also binds to FAM111A known to contribute to viral gene expression, host range restriction and adenovirus replication. Except for JCPyV and BKPy V, all the human polyomaviruses LT-ag's contain much shorter C-terminal region with little homology among them or with SV40.

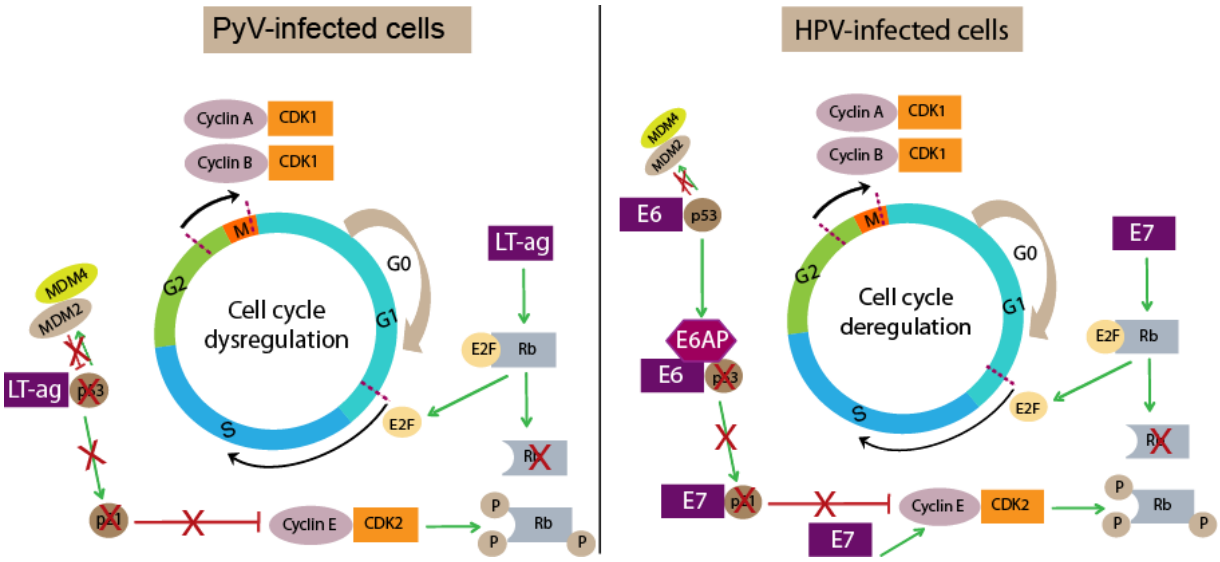


Mechanism of DNA unwinding by the LT-ag. Adapted from (Trakselis and Graham, 2012). LT-ag binds to the origin of replication as a double hexamer which then splits into two single hexamers. Each hexamer tracks along a "leading" strand in the 3'-to-5' direction while excluding the other "lagging" strand, unwinding double-stranded DNA as they go. If the hexamers encounter a protein covalently attached, they can bypass it efficiently without dissociating from the DNA (Yardimci et al., 2012).

Box 5

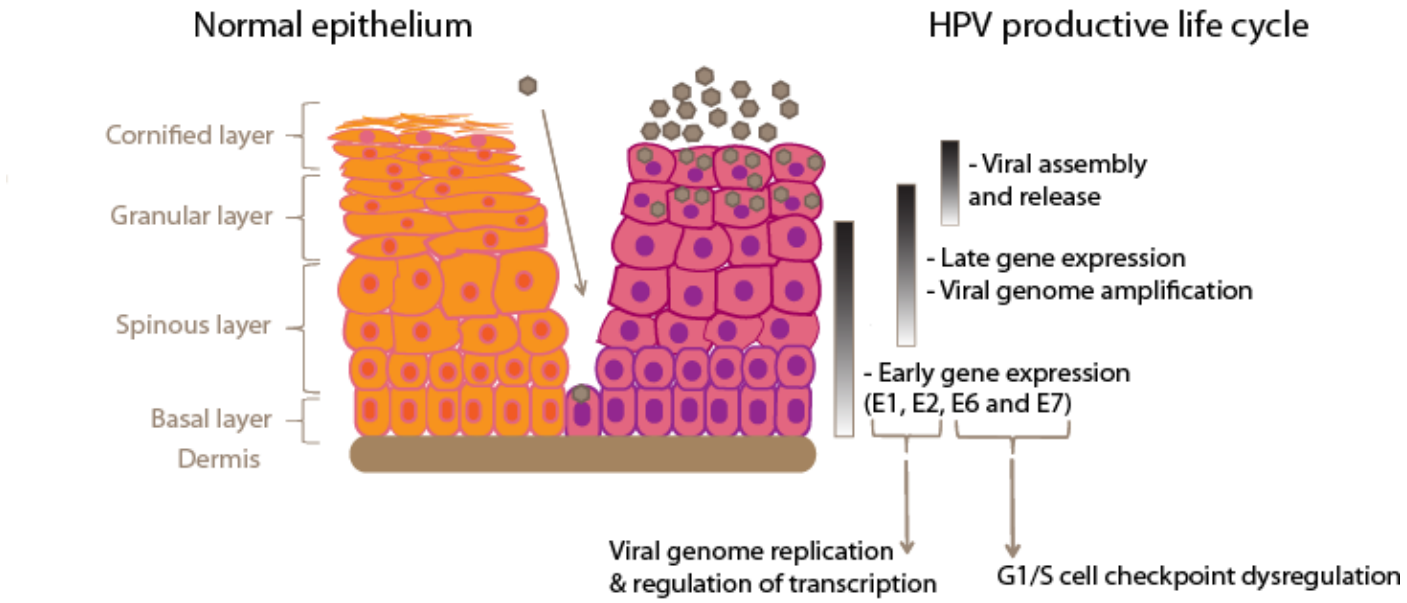


Cell cycle regulation. The transcription factor p53 responds to diverse stresses, including DNA damage, regulating many target genes that induce cell-cycle arrest, apoptosis, senescence, DNA repair or altered metabolism. The tumor suppressor protein p53 prevents cells from entering to or progressing through the cell cycle under conditions that could cause DNA damage. p53-functions in the cell cycle include the control of the G1 transition to the S phase of the cell cycle at the G1 checkpoint by inducing expression of cyclin inhibitors p16, p21 and p27 that block the activities of cyclin-cyclin-dependent kinase (CDK) complexes, thus mediating arrest of the cell cycle by blocking the progression of the cell cycle at the G1/S transition. Because of the central role of p53 in life or death decisions, an exquisite control mechanism of p53 exists. Central to this regulation are the essential p53 inhibitors MDM2 and MDM4, although other participants in p53 control have been described although they are not shown in this figure. In normal cells, Mdm2 and Mdm4 (also called Mdmx; human orthologues often referred to as HDM2 and HDM4/HDMX, respectively) are key regulators of the tumor suppressor protein p53 (MDM2 mainly regulates p53 stability while MDM4 has a major role in regulating p53 activity). DNA damage induces MDM2 self-degradation and an MDM2-dependent degradation of MDM4, a process essential to mount a p53 response.



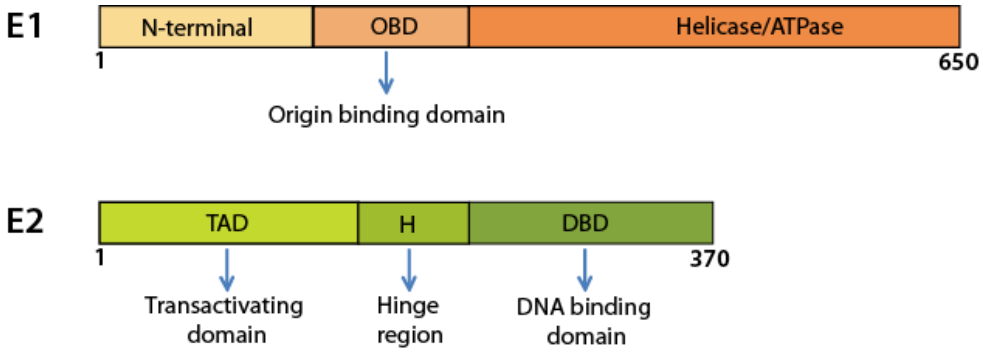
Deregulation of the cell cycle by polyoma- and papillomaviruses. Initially, polyomavirus LT-ag binds to products of the RB-family of proteins thereby interfering with their activity and inducing the infected cell to enter the cell cycle S phase. Subsequently, the LT-ag inactivation of p53 allows re-phosphorylation of Rb proteinsthrough the cyclin dependent kinase (cdk) pathway and prevents the p53-mediated apoptosis of the infected cell. In HPV, Inactivation of p53 by the high-risk E6 proteins results in abrogation of growth arrest and pro-apoptotic effects of p53 whose levels increase due to the constitutive E2F gene transcription as a consequence of inactivation of Rb proteins by the E7 oncoprotein.

Box 6

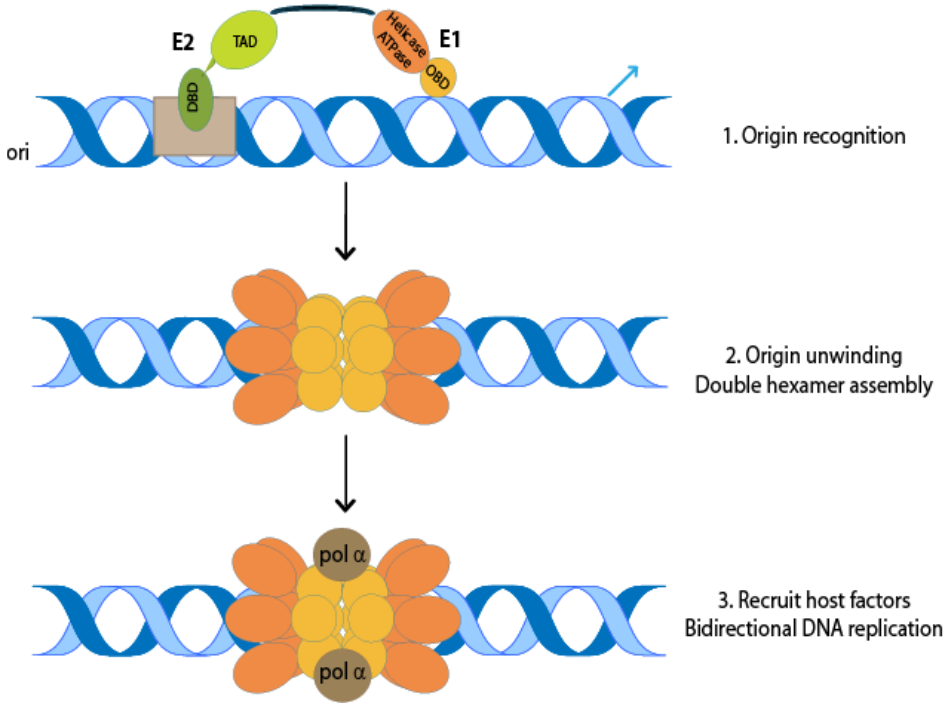


Scheme of the life cycle of human papillomaviruses. Adapted from (Moody and Laimins, 2010). Following exposure through microwounds, keratinocytes in the basal layer of the epithelium are infected by HPV. The infected epithelium (on the right) is compared to the uninfected epithelium (on the left). Following infection, viral genomes are maintained in the nucleus as low copy numbers in an episomal state and they are replicated together with the cellular DNA. Early viral genes are expressed. Following cell division, one daughter cell leaves the basal layer undergoing differentiation and this process of differentiation induces the productive phase of the viral life cycle. In order to activate the cellular DNA synthesis machinery, the viral E6 and E7 proteins are expressed which are responsible for deregulation of the cell cycle control, pushing the differentiating cells to enter into S phase of the cell cycle. Viral genome replication occurs then in cells that normally would have undergone terminal differentiation. The late proteins L1 and L2 are synthesized only in the upper layers of the epithelium and are assembled together with the viral genomes to form virions that are released at the surface of the epithelium.

Box 7

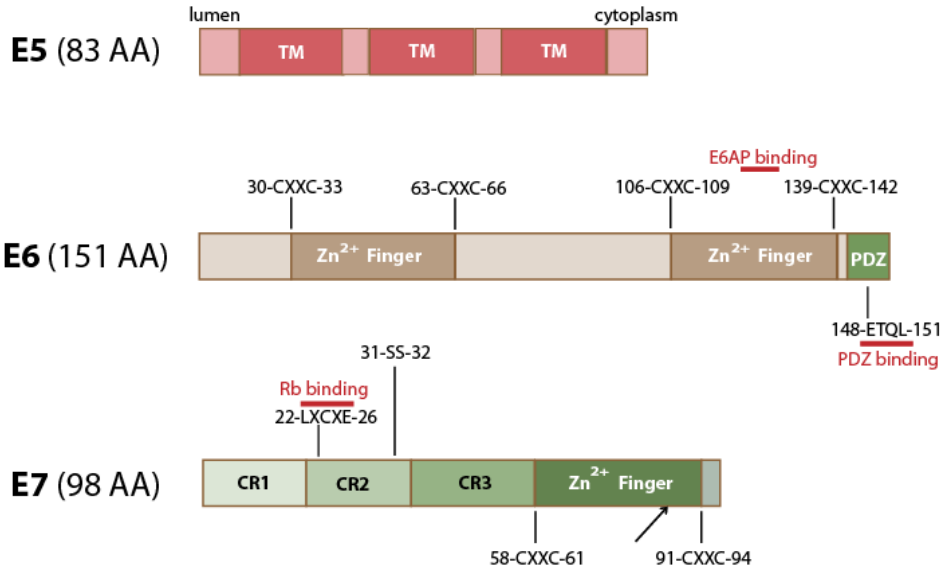


Schematic representation of E1 and E2 proteins. Adapted from (D'Abramo and Archambault, 2011). E1 and E2 are about 650 and 370 amino acids long and are necessary for replication of the viral genome. Three functional domains are recognized in E1: a C-terminal ATPase/helicase domain that can oligomerize into hexamers, a central origin DNA-binding domain (OBD), and an N-terminal regulatory region which is essential for optimal replication in vivo but not in vitro. The N-terminal region consists of conserved sequences for nuclear localization (NLS), nuclear export (NES) and a conserved cyclin-binding motif. E1 functions as a DNA binding protein to recognize the viral origin and also as a helicase to unwind DNA. E1 is also responsible for the recruitment of cellular proteins to the origin of replication. The E2 protein contains two functional domains: an N-terminal transactivation domain (TAD) that is involved in transcriptional regulation and direct association with E1, and a C-terminal DNA-binding/dimerization domain (DBD). These domains are separated by a hinge region. E2 facilitates recognition of the viral replication origin and helps in the assembly of additional E1 proteins into replication competent double hexamers required for bidirectional DNA unwinding. E2 is also implicated in the regulation of viral gene transcription and segregation of the viral genome at mitosis.



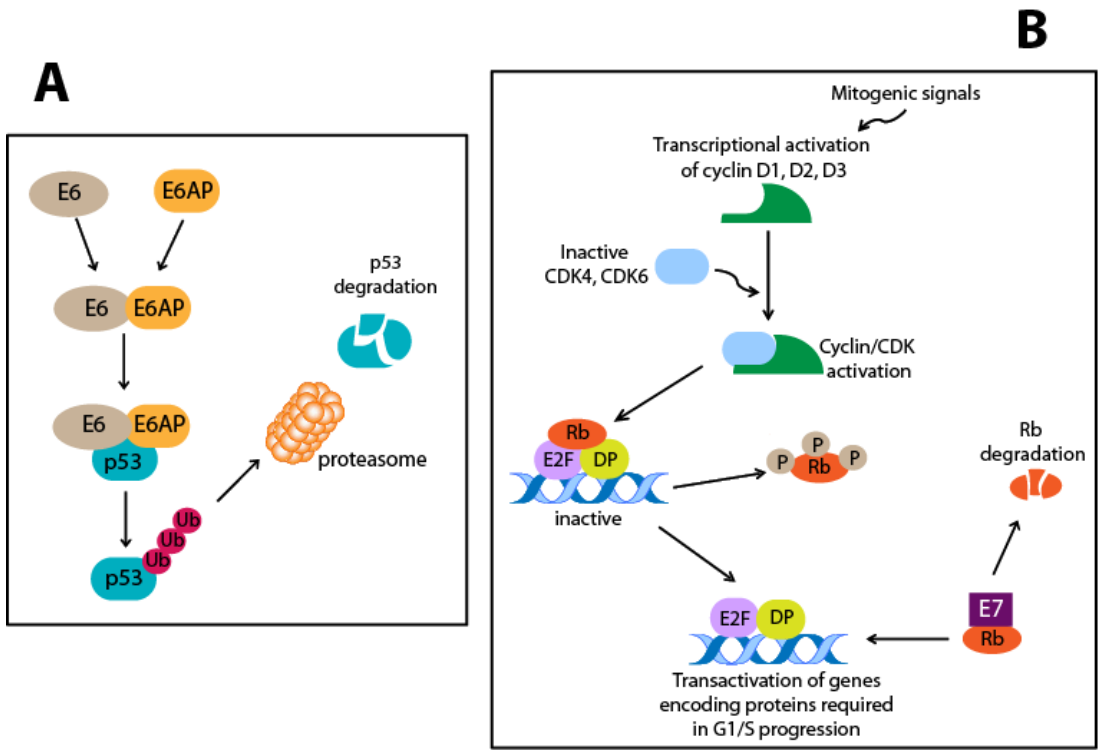
Initiation of HPV DNA replication. Replication begins with the recruitment of E1 by E2 to the Ori (which contains of a cluster of three E2 binding sites flanking a series of overlapping E1 binding sites) (Chow and Broker, 2013). This recruitment step involves an essential protein-protein interaction between the E2 transactivating domain (i.e. TAD) and the E1 helicase domain. In a second step, the E2 protein recruits additional E1 molecules promoting their assembly into a replication-competent double hexameric helicase. ATP also stimulates the oligomerization of E1 and is required for the helicase activity of E1 protein. Finally, the E1 protein interacts with host cell replication factors [replication protein A, topoisomerase I and DNA polymerase α primase (pol α)] promoting bidirectional replication of the viral genome. Following initiation, the clamp loader, RFC (replication factor C

Box 8



Schematic representation of high-risk HPV16 E5 (top), E6 (middle) and E7 (bottom) oncoproteins. C: cysteine, L: leucine, E: glutamic acid, T: threonine, Q: glutamine, S: serine, X: any amino acid. TM: transmembrane domain, CR: conserved region. E5 is a transmembrane protein that functions by inducing ligand-independent dimerization and activation of receptor protein tyrosine kinases including epidermal growth factor receptor. E5 oncoprotein may contribute to some early steps of viral transformation but it is not necessary for malignant progression and/or maintenance of the transformed phenotype and it is generally not expressed in cervical carcinoma. In contrast to E5, the E6 and E7 oncoproteins are consistently expressed in cervical carcinomas and maintenance of the transformed state. Both E6 and E7 contain zinc-binding domains consisting of two copies of CXXC separated by 29 amino acid residues and they may have evolved from a common ancestor.

Box 9



(A) Targeting of the tumor suppressor protein p53 via ubiquitination by HPV E6. The best characterized HPV16 E6 activity is the association with the ubiquitin-protein ligase E6AP (E6 associated protein). The dimeric complex then binds to p53 and E6AP catalizes the multi-ubiquitination of p53. **(B) Deregulation of the cell cycle restriction point G1/S by HPV E7.** The E7 protein binds to several cellular factors, being the best characterized of these interactions the association with the retinoblastoma (Rb) family of proteins. The Rb family of proteins controls the G1/S phase transition by regulating the activity of the E2F family of transcription factors. The transcriptionally active forms of E2F are heterodimers that contain an E2F polypeptide (E2F1-8) and a polypeptide encoded by the E2F dimerization partner (DP) gene family (DP-1, DP-2). The transcriptional activity of E2F/DP heterodimers is influenced by association with the members of the retinoblastoma (Rb) tumor suppressor protein family (pRb, p107, and p130). E2F transcription factors are critical regulators of G1 exit and S-phase progression as well as a number of other cellular processes, including cellular differentiation, apoptosis, and genomic instability. The G1 specific Rb/E2F complex acts as a transcriptional repressor. In quiescent cells, the hypo-phosphorylated form of Rb represses transcription of E2F-dependent promoters by directly binding to the E2F transactivation domain. Following exposure to mitogenic signals, normal cells activate genes encoding the G1 specific D-type cyclins (i.e. D1, D2, and D3). These cyclins associate with a catalytic subunit (i.e. CDK4 or CDK6) and the kinase complexes phosphorylate Rb in mid-G1 phase which results in the release of active E2F/DP-1 heterodimer complexes and progression through the restriction point. The binding of high-risk E7 to Rb disrupts Rb-E2F complexes, leading to the constitutive expression of E2F-reponsive genes. As a result, cells that express E7 can enter the S phase without mitogenic signals.